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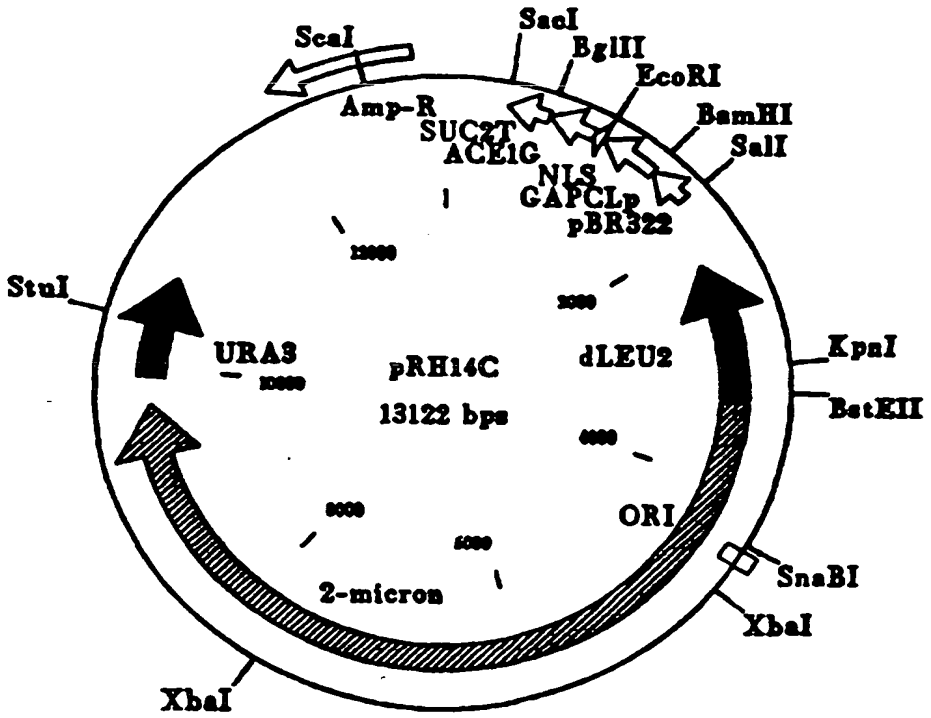

(57) Abstract

Described is a dual-hybrid approach for the determination of interactions between a phosphatase or a kinase and polypeptides that bind to these enzymes like autoinhibitory domains. Using the inventive approach it is possible to measure the influence of antagonists and agonists and, hence, to design regulatory polypeptides with increased regulatory properties.

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DUAL HYBRID SYSTEM

Phosphatases and kinases are enzymes that are generally involved in the regulation of enzyme activity by adding or removing of phosphate groups to enzymes. The activity of these enzymes is often regulated by the binding of another protein and/or by an autoinhibitory domain. This binding of the autoinhibitory domain again is regulated, e.g., by a peptidic or non-peptidic compound that binds to the autoinhibitory domain.

A protein phosphatase of the 2B subfamily, one of four classes of phosphoserine/ phosphothreonine-specific phosphoprotein phosphatases that have been distinguished on the basis of their sensitivities to inhibitors and divalent cations, is calcineurin. Calcineurin is the only known phosphatase that is regulated specifically by Ca^{2+} and calmodulin. The calcineurin holoenzyme purified from mammalian cells (Guerini *et al.*, Proc. Natl. Acad. Sci. USA (1989), **86**, 9183-9187) is a heterodimer consisting of a large catalytic subunit (51-61kD) and a small regulatory subunit (~19kD). The catalytic subunit binds calmodulin, and the regulatory subunit attaches up to four molecules of Ca^{2+}. The catalytic activity of the calcineurin holoenzyme is completely inhibited by a synthetic peptide corresponding to a region in the C-terminus of the catalytic subunit in the presence of Ca^{2+}-calmodulin. This suggests that the catalytic subunit contains an autoinhibitory domain (AID) (Hashimoto *et al.*, J. Biol. Chem. (1990), **265**, 1924-1927).

The budding yeast *Saccharomyces cerevisiae* contains proteins with properties similar to the mammalian catalytic and regulatory subunits of calcineurin. In fact, yeast cells possess two genes, CNA1 and CNA2, which encode for the mammalian catalytic subunit (Cyert *et al.*, Proc. Natl. Acad. Sci. USA (1991), **88**, 7376-7380). Both the Cna1 and Cna2 proteins contain regions which have similarity to the putative AID sequence of the mammalian enzyme (Hashimoto *et al.*, J. Biol. Chem. (1990), **265**, 1924-1927).

Recent findings indicate that two drug-protein complexes, cyclosporin A-cyclophilin and FK506-FKBP, probably bind to the same target, calcineurin (Schreiber, Cell (1992), **70**, 365-368).

Cyclosporin A (CsA) is an undecapeptide (Schreiber, Science (1991), **251**, 283-287) and acts as an inhibitor of T-cell activation. It is currently the favored therapeutic agent for prevention of graft rejection after organ and bone marrow transplantation. CsA binds to family of conserved proteins, named cyclophilins, having high affinity for CsA (Schreiber, Science (1991), **251**, 283-287).

More recently, the new compound FK506 (a macrolide; Liu, Immunol. Today (1993), **14**, 290-295) has been discovered that also inhibits T-cell activation. For FK506 it has been shown that this compound binds to a separate group of conserved proteins (Schreiber, Science (1991), **251**, 283-287), which have been termed the FK-binding proteins (FKBPs).

An example for a cAMP dependent protein kinase is PKA. This enzyme is a member of the large group of eukaryotic protein kinases that play critical roles in regulation of cell growth and comprises a catalytic and a regulatory subunit. PKA in its inactive state consists of a tetramer consisting of two identical catalytic and two identical regulatory subunits. This enzyme is activated, like the other protein kinases, by dissociation of the regulatory and the catalytic subunit to form a dimer of active catalytic subunits. This dissociation is mediated, e.g., by cAMP.

Another example for a kinase is p70S6, a single chain enzyme involved in the regulation of protein synthesis. The target for p70S6 is the 40S ribosomal protein S6. It is supposed that phosphorylation of S6 either triggers or facilitates the activation of protein synthesis. p70S6 is supposed to be regulated by an autoinhibitory domain at the C-terminus (Flotow *et al.*, J. Biol. Chem., (1992), **267**, 3074-3078) and through serine/threonine phosphorylation by insulin/mitogen-activated protein kinases.

A new approach for the detection of protein-protein interaction, as for example, crucial in the regulation of protein phosphatases or kinases is the two-hybrid system (Fields et al., Nature (1989), **340**, 245-246). This system provides a convenient method in which the possible interactions between a peptide and a protein can be examined in a cellular environment. The system depends on the reconstitution of a transcription factor from its two essential components, the DNA-binding domain and its activating sequence. In particular, two fusion proteins are generated, each comprising one of the two domains of

the transcription factor and one of the two proteins that are being tested for their ability to bind to each other. Only when the proteins to be tested interact, a functional transcription reconstitutes. Depending on the strength of the protein-protein interactions, the activation of gene transcription varies. To monitor the formation of a functional transcription factor, the expression of the gene triggered by said reconstituted transcription factor is measured.

Summary of the Invention

Surprisingly, it has now been found, that this dual-hybrid approach is capable of determining the interaction between a phosphatase or a kinase and its autoinhibitory domain (AID). Using the inventive approach it is surprisingly possible to measure the influence of antagonists and agonists and, hence, to find new regulatory polypeptides and to design regulatory polypeptides with increased regulatory properties. It has been surprisingly found, for example, that cyclosporin A and FK506 despite their different structure inhibit the formation of active-center-AID-complex. Using the inventive method it is surprisingly possible to identify new agonists or antagonists of a phosphatase or a kinase. Additionally, it has been found surprisingly that autoinhibitory domains exhibit better binding properties when present in several copies.

Detailed description of the invention

The current invention concerns a transcription system for measuring the interaction between a phosphatase or kinase, or a mutein or fragment thereof that binds an autoinhibitory domain, with said autoinhibitory domain, comprising

a) the DNA binding domain of a transcription factor and the transcription activation domain of a transcription factor that are separated, wherein one of the two transcription factor domains is linked to a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain; and the other of the two transcription factor domains is linked to a polypeptide comprising an autoinhibitory domain that is capable of binding to the polypeptide linked to the first transcription factor domain, and

b) a DNA that is transcribed when the DNA binding domain of said transcription factor and the transcription activation domain of said transcription factor are connected *via* the polypeptides linked thereto.

Using the inventive system it is possible to measure all parameters that influence the binding of the autoinhibitory domain to its target region on the regulated enzyme.

A preferred phosphatase or kinase comprises an autoinhibitory domain (AID) that can be located on the catalytic or a regulatory subunit. Examples for a phosphatase or kinase are phosphatase A1 and A2B, cyclic GMP dependent kinase (cGPK), myosin light chain kinase (MLCK), myosin heavy chain kinase, dsRNA-dependent kinase, dsDNA-dependent kinase, protease activated kinase I and II, cell cycle kinase cdc-28 MPF, growth factor regulated kinase, mammary gland casein kinase, glycogen synthase kinase-3, AMP activated protein kinase, pyruvate dehydrogenase kinase, branched chain α-ketoacid dehydogenase kinase, endogenous eIF-4E kinase, histone H4 kinase I and II, isocitrate dehydrogenase kinase, β-adrenergic receptor kinase, rhodopsin kinase, tropomyosin kinase, proline-dependent protein kinase, calmodulin dependent kinase AII and III, phosphokinase, casein kinase AI and II, pp60, the family of src kinases, the family of cyclin-dependent kinases (cdk-s), EGF receptor, insulin receptor, spleen tyrosine kinase, protein kinase C, calcineurin, PKA and p70S6 kinase. More preferred are calcineurin, PKA and p70S6 kinase.

In the inventive system it is advantageous to use a form of said phosphatase or kinase or a mutein or fragment of said phosphatase or kinase that has a free AID binding domain. In this form the AID that is part of the original enzyme is not bound to the catalytic domain. This form can be achieved *via* phosphorylation, lack of certain deactivating factors, addition of activation factors or *via* a mutein that does not comprise a functional AID. The AID can be inactivated using several approaches. It is, for example, possible to delete the AID on DNA-level, to alter the AID by mutagenesis to be inactive or to use only a fragment of the protein that does not comprise the AID. Nevertheless, it is important that the mutein is not changed to such an extent that the autoinhibitory domain used in the dual hybrid test cannot bind anymore. It is, for example, possible to use a fragment of calcineurin that lacks the C-terminal AID, especially the C-terminal 40-60 amino acids.

Autoinhibitory domains that interact with or bind to a phosphatase or kinase are well known in the art. The interaction of these autoinhibitory domains and the corresponding enzymes by formation of a complex is sensitive to various parameters like compounds that compete with the autoinhibitory domain for the same attachment site, compounds that change the

affinity of the autoinhibitory domain for the attachment site or compounds that change the affinity of the attachment site for the autoinhibitory domain. These compounds can be, for example, peptides, chemical compounds, metal salts or compounds comprising peptidic and non-peptidic parts. Examples for known regulatory compounds are DNA, RNA, steroids, macrolides, cAMP, cGMP, calmodulin, phosphatidyl serine, diacyl glycerol, insulin, epidermal growth factor, interleukins, cytokines, growth factors like IGF and GMCSF, and Ca^{2+}. Preferred examples for such autoinhibitory domains are the inhibitory or auto-inhibitory domains of the catalytic or regulatory subunit of a phosphatase or kinase (Kemp *et al.*, Trends in Biochem. Sci. (1990), 15, 342-346), e.g. of the regulatory subunit of PKA and the AID of calcineurin or p70S6 and fragments thereof that are still capable of binding to the phosphatase or kinase in question. In a more preferred embodiment of the invention the autoinhibitory domain and the fragment that binds an autoinhibitory domain originate from the same enzyme komplex. In an even more preferred embodiment of the invention the autoinhibitory domain and the fragment that binds an autoinhibitory domain originate from the same enzyme. Also included are artificial substrates or pseudosubstrates of the phosphatase or kinase to be tested.

Pseudosubstrates are, for example, created by replacing amino acids of natural occurring autoinhibitory domains with amino acids that promote binding to the enzyme. Examples are the replacement of one or more serine, threonine and/or thyrosine by another amino acid like alanine (Hardie, Nature (1988), 335, 592-593).

A preferred polypeptide that binds to the phosphatase or kinase as described above is a polypeptide comprising the regulatory subunit of PKA, the autoinhibitory domain of calcineurin and/or p70S6 kinase, as well as fragments thereof capable of binding to enzyme in the test. Especially preferred are the regulatory subunit of PKA, a fragment of calcineurin comprising the C-terminal 40 to 60 amino acid and a fragment of p70S6 kinase comprising amino acid 332 to 502 or 399 to 502.

Additionally, the autoinhibitory domain can be linked to the transcription factor domain as a single copy or in several copies which is preferred, e.g., 1 to 5 copies of the AID or 2 to 4 copies which is preferred. It is also possible to link different autoinhibitory domains to the same transcription factor to test, e.g., different influences. The different autoinhibitory domains or the copies of the same autoinhibitory domain may be connected directly or with

spacer groups. Spacer groups are, for example, α-helix breakers like short peptides of repeated amino acids like Gly_3, Gly_4 or Gly_5.

Transcription factors according to the invention are proteins that bind to a certain region of DNA (promoter) and initiate the transcription of a DNA sequence that belongs to it. These transcription factors usually comprise a domain that is responsible for the DNA recognition or binding and a domain that initiates transcription. DNA recognition and transcription initiation domains are known for several transcription factors such as Ace1, Gal4, VP16, p53 and lexA.

For the inventive system the two domains necessary for transcription initiation of a certain gene can originate from two different transcription factors or from the same transcription factor. For example, both domains may originate form the CUP1 or Gal4 transcription factor or the DNA binding domain originates from the lexA transcription factor and the transcription activating domain originates from the VP16 or p53 transcription factor; or any other combination thereof.

For example, in the case of Ace1, a transcription factor for the initiation of the transcription of metallothioneins (CUP1), the DNA binding domain is located in the region comprising N-terminal amino acids, preferred are the N-terminal 122 amino acids, and the transcription activation domain is located in the region comprising C-terminal amino acids, wherein the C-terminal 100 amino acids are preferred, (Cizewski *et al.*, Proc. Natl. Acad. Sci. USA (1988), 86, 8377-8381).

According to the invention, one of the two transcription factor domains is linked to a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain, as described above. The other of the two transcription factor domains is linked to a polypeptide comprising an autoinhibitory domain that is capable of binding to the phosphatase or kinase linked to the first transcription factor domain, as described above.

These autoinhibitory domains can be linked to one of the transcription factor domains, as defined above, directly or *via* a spacer, in frame.

A further aspect of the invention is an expression cassette for the expression of a fusion protein comprising the DNA binding domain or the transcription activation domain of a transcription factor and
a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain; or
a polypeptide comprising said autoinhibitory domain or an active fragment thereof that is capable of binding to said phosphatase or kinase or a fragment thereof.

Methods for the construction of an expression cassette comprising a DNA coding for a fusion protein comprising one of the two transcription factor domains and one of said polypeptides are known in the art and are described for example in Fields *et al.*, Nature (1989), **340**, 245-246; Chien *et al.*, Proc. Natl. Acad. Sci. USA (1991), **88**, 9578-9582; Yang *et al.*, Science (1993), **257**, 680-682.

Another aspect of the current invention is a DNA that is transcribed when the DNA binding domain of a transcription factor and the transcription activation domain of a transcription factor are joined *via* the polypeptides linked thereto, as described above, if this DNA is not already part of the natural genome of the host that is used for the test. This DNA is usually comprised in an expression cassette comprising a promoter that is operably linked to said DNA and to a sequence containing a transcription termination signal, as described above. In a preferred embodiment of the invention said DNA is coding for a polypeptide.

The promoter of the expression cassette is chosen in accordance with the transcription factor fragment used. For example, suitable transcription factor/promoter combinations are Ace1/CUP1p or Gal4/Gal1-Gal10p (Chien *et al.*, Proc. Natl. Acad. Sci. USA (1991), **88**, 9578-9582; Yang *et al.*, Science (1993), **257**, 680-682). In a preferred embodiment of the present invention the promoter is the CUP1 promoter.

A suitable DNA that is transcribed under the control of this promoter usually causes an effect that can be measured easily during or after transcription or translation. Preferred are transcription or translation products that can be measured easily e.g. *via* the measurement of the amount of protein, mRNA or DNA produced; or that cause an effect that can be

measured easily, e.g. cell growth, an enzymatic reaction or color. Hence, the transcribed DNA codes, for example, for a protein that is produced in an amount that is related to the amount of activation of said promoter and that is, e.g., not produced elsewhere in the chosen microbiological host under the applied conditions. Examples for suitable proteins are the metallothionein that is encoded by the yeast CUP1 gene, β-galactosidase or luciferase. Preferred is metallothionein and β-galactosidase.

An expression cassette for said suitable DNA may be already present in the microbiological host. In this case, the corresponding original transcription factor is inactivated or replaced by the inventive transcription factor fusion constructs. Hence, the reconstitution of the promoter will induce the transcription of a genuine gene, that is, e.g., necessary for survival of the microbiological host under the condition applied. An example is the replacement of the original Ace1 domain(s) with the separated Ace1 domain(s) modified according to the invention, resulting in a yeast whose sensitivity to Cu^{2+} depends on the reconstitution of the functional Ace1. A microbiological host transformed with this inventive system will, for example, grow in Cu^{2+} containing media only if the two transcription factor domains are linked *via* the associated phosphatase or kinase, as defined above, and the associated autoinhibitory domain of this phosphatase or kinase, as defined above.

In the expression cassettes of the present invention, the promoter is operably linked to the encoded protein. Additional sequences, such as pro- or spacer-sequences which may or may not carry specific processing signals can also be included in the constructions to facilitate accurate processing of precursor molecules. Alternatively fused proteins can be generated containing internal processing signals which allow proper maturation in *vivo* or in *vitro*.

Preferably, the expression cassettes according to the present invention comprise also the 3'-flanking sequence of a gene which contains the proper signals for transcription termination and polyadenylation. Suitable 3'-flanking sequences are for example those of the gene naturally linked to the promoter used, or 3'-flanking sequences of other yeast genes, e.g., of the PHO5 or the SUC2 gene. An expression cassette comprising the DNA that is transcribed under the control of the inventive transcription factor construct may, for example, contain additionally a DNA sequence encoding a signal peptide.

The expression cassettes for the transcription factor fusion proteins and the DNA that is transcribed by the reconstituted transcription factor may be present on different plasmids or some or all together on one plasmid. These plasmids may be integrated into the genome of the microbiological host or may retain as separate units. It is for example possible to integrate the expression cassettes for the modified transcription factor domains into the genome of the microbiological host, e.g. at the position of the original transcription factor domains and inactivating therewith the original factor, as described in (Munder & Fürst, Mol. Cell. Biol. (1992), 12, 2091-2099). The expression cassette expressing the polypeptide to be monitored may be inserted, if it is not already part of the genome of the microbiological host, in form of a stable plasmid, e.g., in *Saccharomyces cerevisiae* in form of a two-micron based plasmid as described in EP-341215.

The hybrid vectors according to the invention are selected from the group consisting of a hybrid plasmid and a linear DNA vector and are further selected depending on the host organism envisaged for transformation.

Accordingly, the invention relates also to hybrid vectors comprising the expression cassettes described above.

Hybrid plasmids that retain as separate units carry the expression cassettes according to the invention and as additional DNA sequences a yeast replication origin and a selective genetic marker for yeast. Hybrid plasmids containing a yeast replication origin, e.g. an autonomously replicating segment (Ars), are extrachromosomally maintained within the yeast cell after transformation and are autonomously replicated upon mitosis. Hybrid plasmids containing sequences homologous to yeast 2μ plasmid DNA can be used as well. These hybrid plasmids will get integrated by recombination into 2μ plasmids already present within the cell or will replicate autonomously. 2μ sequences are especially suitable for high-frequency transformation plasmids and give rise to high copy numbers.

In addition, the preferred hybrid plasmids according to the invention may include a DNA sequence as part of a gene present in the host yeast chromosome (e.g. the gene for the transcription factor domain or its promoter linked to the transcription factor constructs). By

virtue of the homologous sequence, which should amount to a stretch of at least 20 to 100 deoxynucleotides, the whole plasmid or linear fragments thereof can be stably incorporated into the host chromosome by recombination. Thus, during propagation the progeny cells will retain the introduced genetic material even without selective pressure.

As to the selective gene marker for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker. Suitable markers for yeast are particularly those expressing antibiotic resistance or, in the case of auxotrophic yeast mutants, genes which complement host lesions. Corresponding genes confer, for example, resistance to the antibiotic cycloheximide or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, HIS3 or TRP1 gene. It is also possible to employ as markers structural genes which are associated with an autonomously replicating segment providing that the host to be transformed is auxotrophic for the product expressed by the marker.

Advantageously, the additional DNA sequences which are present in the hybrid plasmids according to the invention also include a replication origin and a selective genetic marker for a bacterial host, especially *Escherichia coli*. There are useful features which are associated with the presence of an *E. coli* replication origin and an *E. coli* marker in a yeast hybrid plasmid. Firstly, large amounts of hybrid plasmid DNA can be obtained by growth and amplification in *E. coli* and, secondly, the construction of hybrid plasmids is conveniently done in *E. coli* making use of the whole repertoire of cloning technology based on *E. coli*. *E. coli* plasmids, such as pBR322 and the like, contain both *E. coli* replication origin and *E. coli* genetic markers conferring resistance to antibiotics, for example tetracycline and ampicillin, and are advantageously employed as part of the yeast hybrid vectors.

The additional DNA sequences which contain, for example, replication origin and genetic markers for yeast and a bacterial host (see above) are hereinafter referred to as "vector DNA" which together with the yeast promoter and the coding region is forming a hybrid plasmid according to the invention.

The preferred hybrid vectors of the present invention are prepared by methods known in the art, for example by linking a suitable promoter, a coding region, the 3' flanking sequence of a yeast gene and optionally vector DNA.

For the preparation of hybrid plasmids, conveniently mapped circular vector DNA, for example bacterial plasmid DNA or the like (see above), having at least one restriction site, preferably two or more restriction sites, can be employed. Advantageously, the vector DNA already contains replication origins and gene markers for yeast and/or a bacterial host. The vector DNA is cleaved using an appropriate restriction endonuclease. The restricted DNA is ligated to the DNA fragment containing the yeast promoter and to the DNA segment coding for the desired protein. Prior to or after linking of the promoter and the coding region (or simultaneously as well), it is also possible to introduce replication origins and/or markers for yeast or a bacterial host. At all events, the restriction and ligation conditions are to be chosen in such a manner that there is no interference with the essential functions of the vector DNA and of the promoter. The hybrid vector may be built up sequentially or by ligating two DNA segments comprising all sequences of interest.

Various techniques may be used to join DNA segments in vitro. Blunt ends (fully base-paired DNA duplexes) produced by certain restriction endonucleases may be directly ligated with T4 DNA ligase. More usually, DNA segments are linked through their single-stranded cohesive ends and covalently closed by a DNA ligase, e.g. T4 DNA ligase. Such single-stranded "cohesive termini" may be formed by cleaving DNA with another class of endonucleases which produce staggered ends (the two strands of the DNA duplex are cleaved at different points at a distance of a few nucleotides). Single strands can also be formed by the addition of nucleotides to blunt ends or staggered ends using terminal transferase ("homopolymeric tailing") or by simply chewing back one strand of a blunt-ended DNA segment with a suitable exonuclease, such as I exonuclease. A further approach to the production of staggered ends consists in ligating to the blunt-ended DNA segment a chemically synthesized linker DNA which contains a recognition site for a staggered-end forming endonuclease and digesting the resulting DNA with the respective endonuclease.

The components of the hybrid vector according to the invention, such as the yeast promoter, the structural gene optionally including a signal sequence, transcription terminator, the replication system etc., are linked together in a predetermined order to assure proper function. The components are linked through common restriction sites or by means of synthetic linker molecules to assure proper orientation and order of the components.

A linear DNA vector is made by methods known in the art, e.g. linking the above yeast promoter to the coding region in such a manner that the coding region is controlled by said promoter, and providing the resulting DNA with a DNA segment containing transcription termination signals derived from a yeast gene.

Joining of the DNA segments may be done as detailed above, viz. by blunt end ligation, through cohesive termini or through chemically synthesized linker DNAs.

The DNA fragments coding for fragments or domains of polypeptides, e.g., a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain; a polypeptide comprising said autoinhibitory domain; the DNA binding domain; and the transcription activation domain of a transcription factor; as described above, can be isolated by methods known in the art. It is for example possible to isolate the desired fragment from a gene bank using specific hybridization probes, to synthesize the desired fragment in a DNA synthesizer, to amplify the specific fragment via PCR techniques or to use any combination thereof.

Excision of a portion of a DNA coding for a protein-domain like the transcription activation or the DNA binding domain of a transcription factor, or a phosphatase or kinase lacking the autoinhibitory domain may be effected by using restriction enzymes. A prerequisite of this method is the availability of appropriate restriction sites in the vicinity of the ends of the DNA coding for the desired domain. For convenience and in order to facilitate handling of the protein domain the latter is advantageously contained in a greater DNA segment provided with appropriate linkers which allow insertion and cloning of the segment in a cloning vector.

A preferred microbiological host for the constructs described above is a yeast or bacterial cell, wherein yeast is more preferred and *Saccharomyces cerevisiae* is especially preferred.

The expression cassettes or hybrid vectors described above can be integrated into the microbiological host by standard methods in genetic engineering.

The microbiological host originating therefrom comprises one or more expression cassettes for the two modified transcription factors and one ore more expression cassettes expressing the polypeptide to be monitored.

The transcription system according to the invention or the host transformed therewith can be used for the identification of an agonist or antagonist of a phosphatase or kinase as described above.

In a preferred embodiment of the invention the inventive transcription system can be used to identify an antagonist of the phosphatase or kinase as described above.

Accordingly, a further embodiment of the invention is a method for the identification of a phosphatase or kinase agonist and antagonist comprising

a) treating a transformed microbiological host as defined above with a test compound, and
b) measuring the amount of transcription activation caused by the two modified transcription factor fragments, e.g. by measuring the amount of a produced mRNA, DNA, protein or by measuring an effect caused by the induced transcription or translation product.

The agonist and antagonist identified using the inventive system have valuable pharmacological properties an can be used in a method of treatment. Indications for these agonists and antagonists are, for example, cancer, arthritis, psoriasis, graft rejection, autoimmune diseases, allergy, Alzheimer's disease and regulation of cell proliferation. It is, for example, possible to use the calcineurin antagonists in a method of treatment that is characterized by inhibiting T-cell activation, especially in a method of treatment or prevention of graft rejection.

Also enclosed by the present invention is a pharmaceutical composition comprising one or more of the compounds identified by the inventive system or pharmacologically acceptable salt thereof, optionally together with pharmacologically suitable carrier.

Brief description of the drawings

In the following experimental part various embodiments of the present invention are described with reference to the accompanying drawings in which:

Fig. 1 is a schematic illustration of plasmid pRH1.

Fig. 2 is a schematic illustration of plasmid pRH14C.

Fig. 3 is a schematic illustration of plasmid YipCL.

Fig. 4 is a schematic illustration of plasmid pMH7.

Fig. 5 is a schematic illustration of plasmid pMH10.

The following examples illustrate the invention and should not be construed as a limitation thereof.

Examples

All standard methods in genetic engineering are carried out as described in: *Sambrook et al.*, Molecular Cloning: A laboratory manual, 2nd Edn. 1989,

The enzyme employed for all PCR reactions is Vent polymerase (New England Bio-Labs)
The Primers are synthesized on a DNA synthesizer

Example 1: Isolation of yCNA1 and yCNA2

yCNA1 and yCNA2 are the complete genes from the yeast genome encoding the two isozymes for the catalytic subunit of yeast calcineurin.

In order to obtain the yCNA1 and yCNA2 genes for convenient cloning in a yeast expression vector, the polymerase chain reaction (PCR) is performed on yeast genomic DNA (obtained from the wild type strain S288C), using two pairs of deoxyoligoribonucleotides as primers (SEQ ID NOs 1, 2 and 3, 4). The primers correspond to the published sequences of yeast CNA1 and CNA2 (Cyert *et al.*, Proc. Natl. Acad. Sci. USA (1991) 88, 7376-7380). The yeast cDNA is amplified in 30 cycles of PCR. The BglII-HindIII fragments containing the coding regions of yCNA1 and yCNA2 are ligated to the plasmid pUC19Bgl, completely digested with BglII/HindIII. An aliquot of the ligation mixture is added to calcium-treated, transformation-competent *E. coli* HB101 cells. 6ampicillin resistant *E. coli* transformants from each of the two transformations are grown in the presence of 100 mg/l ampicillin. Plasmid DNA is prepared and analyzed by digestion with BglII/HindIII, BglII/BstEII and HindIII/BstEII. The two correct clones with the expected fragments are named pUC19Bgl/BglII-HindIII/yCNA1 and pUC19Bgl/BglII-HindIII/yCNA2, respectively. The DNA inserts in the two plasmids are confirmed by sequencing (using the Applied Biosystems DNA sequencer 370A).

The vector pUC19Bgl, used for the above ligations, is a modified pUC19 (Boehringer Mannheim GmbH, Germany). pUC19 is digested with the restriction enzyme BamHI and the sticky ends are flushed with the large fragment of Klenow polymerase. The blunt ended DNA is ligated with BglII linkers (double-stranded octamers, the sense strand being 'CAGATCTG'; Boehringer), digested with BglII and religated. An aliquot of the ligation mixture is transformed in HB101 cells. Plasmid DNA is prepared from 6 HB101 transformants and are analyzed by digestion with ScaI/BglII and ScaI/BamHI. One correct clone with the expected fragments is named pUC19Bgl. The BamHI and BglII restriction sites are both present in the vector pUC19Bgl. The plasmid pUC18Bgl is prepared in an identical manner from pUC18.

Example 2: Construction of yCNA1Δ and yCNA2Δ, C-terminal truncated versions of the genes encoding the isozymes (i.e. yCNA1 and yCNA2) for the catalytic subunit of yeast calcineurin

In order to obtain the above genes, PCR is performed on the complete yeast CNA1 and CNA2 (pUC19Bgl/ BglII-HindIII/ yCNA1 and pUC19Bgl/BglII-HindIII/yCNA2, as templates;

see Example 1), using two pairs of deoxyoligoribonucleotides as primers (see SEQ ID NOs 1, 5 and 2, 6). The yeast cDNA is amplified as described in Example 1. Two 1670 bp and 1530 bp BglII-HindIII fragments, encoding yCNA1Δ and yCNA2Δ (i.e. yCNA1 and yCNA2 lacking their putative autoinhibitory domains), are isolated. After digestion with BglII/HindIII, the fragments are ligated to the plasmid pUC19Bgl (see Example 1). DNA obtained from transformants are analyzed as described in Example 1). The two correct clones with the expected fragments are named pUC19Bgl/BglII-HindIII/yCNA1Δ and pUC19Bgl/BglII-HindIII/yCNA2Δ, respectively. The truncated gene fragments are confirmed by sequencing, as in Example 1.

Example 3: Construction of y1(AID) and y2(AID), the gene fragments encoding the autoinhibitory peptides of yeast calcineurin yCNA1 and yCNA2

The BglII-XbaI autoinhibitory sequences, y1(AID) and y2(AID) (Cyert *et al.*, Proc. Natl. Acad. Sci. USA (1991), 88, 7376-7380), are obtained by PCR using pUC19Bgl/BglII-HindIII/yCNA1 and pUC19Bgl/BglII-HindIII/yCNA2, as templates (see Example 1). The primers employed are depicted in SEQ ID NOs 7, 8 and 9, 10. The BglII-XbaI fragments are subcloned in pUC19Bgl. The two clones with correct inserts are named pUC19Bgl/BglII-XbaI/y1(AID) and pUC19Bgl/BglII-XbaI/y2(AID), respectively.

Example 4: Isolation of the yeast SUC2 terminator fragments

The SUC2 gene (Taussig *et al.*, Nucleic Acids Res. (1983), 11, 1943-1954) terminator is isolated as four unique fragments (~300 bp) by PCR, using yeast genomic DNA as template: (i) an EcoRI-SacI fragment with primers in SEQ ID NOs 11 and 12; (ii) an XbaI-SacI fragment with primers in SEQ ID NOs 13 and 12; (iii) a BglII-SacI fragment with primers in SEQ ID NOs 14 and 12; (iv) an EcoRI-KpnI fragment with primers in SEQ ID NOs 11 and 15. All the three fragments are subcloned in pUC19 or pUC19Bgl and the DNA inserts are confirmed by DNA sequencing (see Example 1). The resulting plasmids are:

(i) 19/EcoRI-SacI/SUC2t
(ii) pUC19/XbaI-SacI/SUC2t
(iii) pUC19/BglII-SacI/SUC2t
(iv) pUC19/EcoRI-KpnI/SUC2t

Example 5: Construction of pRH10C, which encodes the GAPCL promoter fused to a nuclear localization signal of the SV40 large T antigen which, in turn, is linked to the transcription-activation domain of ACE1 (i.e. ACE1C)

A 393 bp truncated version of the constitutive glyceraldehyde dehydrogenase promoter (GAPCLp) is cloned as a ~670 bp SalI-EcoRI fragment (Meyhack *et al.*, in Hershberger, Queener, Hegeman (ed.), Genetics and molecular biology of industrial microorganisms, 1989, p. 311-321. American Society for Microbiology, Washington, DC) into pBluescriptIIKS+ vector (Stratagene), yielding the plasmid pRH2. The ~670 bp SalI-EcoRI insert in pRH2 contains a 276 bp SalI-BamHI fragment from pBR322 upstream of the GAPCLp.

A 80 bp EcoRI-SpeI double-stranded DNA linker (see SEQ ID NO 16), encoding the nuclear localization signal (NLS) from the simian virus 40 Tantigen (Kalderon *et al.*, Cell (1984), **39**, 499-509; and Nelson *et al.*, Mol. Cell. Biol. (1989), **9**, 384-389), is subcloned in pRH2 and the DNA insert is confirmed by sequencing (see Example 1). One correct clone is referred to as pRH3.

The ~304 bp XbaI-BglII ACE1C transcriptional activation domain from the yeast transcriptional activator ACE1 (Munder *et al.*, Mol. Cell. Biol. (1992), **12**, 2091-2099) is isolated as a PCR product using two primers (see SEQ ID NOs 17 and 18) with the plasmid pTM4 as template (Munder *et al.*, Mol. Cell. Biol. (1992), **12**, 2091-2099). The XbaI-BglII fragment is subcloned in pUC19Bgl. The insert is confirmed by sequencing (see Example 1) and it contains two extra nucleotides after the last complete codon at the 3' end of the gene fragment (see SEQ ID NO 18). The plasmid with the correct insert is named pUC19Bgl/XbaI-BglII/ACEc_3.

The ~670 bp SalI-EcoRI fragment from pRH2 and the ~304 bp XbaI-BglII fragment from pUC19Bgl/XbaI-BglII/ACEc_3 are subcloned in pUC19Bgl completely digested with SalI and BglII. Correct clones are confirmed by appropriate restriction enzyme digests. One such clone is referred to as pRH10C.

Example 6: Construction of yeast expression vectors for the expression of ACE1C-y1(AID) and ACE1C-y2(AID):

y1(AID) and y2(AID) encode the autoinhibitory peptides of the yeast calcineurin catalytic subunits yCNA1 and yCNA2.

The ~1075 bp SalI-BglII fragment from pRH10C (see Example 5), the BglII-XbaI fragment from either pUC19Bgl/BglII-XbaI/y1(AID) or pUC19Bgl/BglII-XbaI/y2(AID) (see Example 3) and the ~300 bp XbaI-SacI fragment from pUC19/XbaI-SacI/SUC2t (see Example 4) are ligated to the vector pDP34 which has been completely digested with SalI and SacI. The plasmid pDP34 is an *E. coli-S. cerevisiae* shuttle vector, which contains the complete *S. cerevisiae* 2-micron plasmid and encodes the *S. cerevisiae* URA3 and dLEU2 genes as yeast selection markers (Meyhack *et al.*, in Hershberger-CL; Queener-SW; Hegeman-G (ed.), Genetics and molecular biology of industrial microorganisms, 1989, p. 311-321. American Society for Microbiology, Washington, DC). After transformation in *E. coli* HB101, DNA is prepared from 12 transformants. Analyses with SalI/SacI and BamHI-SacI confirm the correct clones. Two such clones are referred to as pMH10 [encoding y1(AID)] and pCS100 [encoding y2(AID)].

A control plasmid, which contains only NLS-ACE1C under the control of the GAPCLp, is prepared by ligating the ~1075 bp SalI-BglII fragment from pRH10C (see Example 5) and the ~300 bp BglII-SacI fragment from pUC19/BglII-SacI/SUC2t (see Example 4) to the vector pDP34 which has been completely digested with SalI and SacI. The plasmid is named pRH14C (Fig. 1).

Example 7: Construction of a yeast expression vector for the expression of ACE1C-y1(AID)-$(Gly)_4$-y1(AID) and ACE1C-y2(AID)-$(Gly)_4$-y2(AID), encoding a fusion between the transcription-activation domain ACE1C and duplicate fragments of the autoinhibitory domains of yCNA1 and yCNA2

The ~1075 bp SalI-BglII fragment from pRH10C (see Example 5) and the BglII-XbaI fragments from either pUC19Bgl/BglII-XbaI/y1(AID) or pUC19Bgl/BglII-XbaI/y2(AID) (see Example 3) are ligated to pUC19 which has been completely digested with SalI and XbaI. The two correct clones are named pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y1(AID) or pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y2(AID).

Unphosphorylated linkers (encoding a stretch of four glycines) with SEQ ID NO 19 are ligated to XbaI digested pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y1(AID) or pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y2(AID). The ligated molecules are isolated on a 1% agarose/Tris-acetate gel. The fragments are isolated, purified by GeneClean (Bio 101, CA, USA) and re-annealed by incubating at 95°C followed by slow cooling to room temperature. The DNA obtained from HB101 transformants are analyzed on a 2% agarose gel. The clones which show a distinct increase in the molecular size of the BglII-XbaI fragments from the ones obtained from the starting plasmids (see above, this Example) are named pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y1(AID)-$(Gly)_4$ and pUC19/SalI-XbaI/ GAPFLp-NLS-ACE1C-y2(AID)-$(Gly)_4$.

A second copy of y1(AID) is isolated as a SpeI-HindIII fragment by PCR, using the primers in SEQ ID NOs 20 and 21 and the plasmid pUC19/SalI-XbaI/ GAPFLp-NLS-ACE1C-y1(AID) as template. This is subcloned along with a HindIII-SacI yeast PHO5 transcriptional terminator fragment in pBluescriptKS+. One correct clone is referred to as pBluescriptKS+/SpeI-SacI/y1(AID)-PHO5t.

A second copy of y2(AID) is isolated as a SpeI-SacI fragment by PCR, using the primers in SEQ ID NOs 22 and 12 and the plasmid pCS100 (see Example 6) as template. This is subcloned in pBluescriptKS+. One correct clone is referred to as pBluescriptKS+/SpeI-SacI/y2(AID)-SUC2t.

The ~1100 bp SalI-XbaI fragment from pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y1(AID)-$(Gly)_4$ and the SpeI-SacI fragment from pBluescriptKS+/SpeI-SacI/y1(AID)-PHO5t are ligated to the vector pDP34 which has been completely digested with SalI and SacI. After transformation in *E. coli* HB101, DNA is prepared from 12 transformants. Analyses with SalI/SacI and BamHI-SacI confirm the correct clones. One such clone is referred to as pMH11 (encoding two copies of y1(AID) linked by a glycine linker).

The ~1100 bp SalI-XbaI fragment from pUC19/SalI-XbaI/GAPFLp-NLS-ACE1C-y2(AID)-$(Gly)_4$ and the SpeI-SacI fragment from pBluescriptKS+/SpeI-SacI/y2(AID)-SUC2t are ligated to the vector pDP34 which has been completely digested with SalI and SacI. As

above, analyses with SalI/SacI and BamHI-SacI confirm the correct clones. One such clone is referred to as pCS101 (encoding two copies of y2(AID) linked by a glycine linker).

Example 8: Construction of hCNRA2 and hCNRA2Δ, the complete and truncated genes encoding the catalytic subunit of human calcineurin

The 3' end (~1280bp) of the human gene (Guerini *et al.*, Proc. Natl. Acad. Sci. USA (1989) 86, 9183-9187) is isolated as two fragments (a ~563 bp NcoI-SacI fragment and a ~713 bp SacI-EcoRI fragment) from human fetal brain cDNA library (Stratagene) by PCR, using two pairs of primers with SEQ ID NOs 23, 24 and 25, 26. They are subcloned in the modified pUC19Bgl vector pUC19bg_nc, in which an NcoI site has been introduced (with NcoI linkers; Boehringer) at the SmaI site of pUC19Bgl (see Example 1). Four individual clones with ~1280 bp inserts are sequenced (using the Applied Biosystems DNA sequencer 370A). One correct clone is named pUC19bg_nc/NcoI-EcoRI/3'hCNRA2.

The ~300 bp 5' end (a BglII-NcoI fragment) of hCNRA2 is constructed with two overlapping deoxyoligoribonucleotides (which are chemically synthesized using yeast-biased codons; see SEQ ID NOs 27 and 28). First, primer extension is performed which is followed by a PCR with primers (see SEQ ID NOs 29 and 30; which code for the sense and the anti-sense strand of the 5' and 3' ends of the ~300 bp fragment). The BglII-NcoI fragment is subcloned in pUC19bg_nc (see above; this Example). Six individual clones with ~300 bp inserts are sequenced (using the Applied Biosystems DNA sequencer 370A). One correct clone is named pUC19bg_nc/BglII-NcoI/5'hCNRA2.

The complete hCNRA2 gene is assembled from the chemically synthesized ~300 bp BglII-NcoI fragment and the ~1280 bp NcoI-EcoRI fragment by subcloning in pUC18Bgl (constructed in the way pUC19Bgl is made; Example 1). Six individual clones are analyzed by restriction enzyme digests. One correct cloned is named pKO10.

The complete hCNRA2 gene (but lacking the DNA encoding the 2 amino acids at the N-terminus, Met^1, Ala^2) is isolated as a BglII-EcoRI fragment by PCR, using primers with SEQ ID NOs 31 and 32 and pKO10 as template.

The gene encoding the C-terminal truncated version of hCNRA2 (i.e. hCNRA2Δ), which lacks the autoinhibitory domain (and also the DNA encoding the N-terminal residues, Met^1, Ala^2), is isolated as a BglII-EcoRI fragment by PCR, using primers with SEQ ID NOs 31 and 33 and pKO10 as template.

Example 9: Construction of two yeast expression vectors for the expression of ACE1C-h(AID) and ACE1C-h(AID)-$(Gly)_4$-h(AID): h(AID) encodes the autoinhibitory peptide of the human brain calcineurin catalytic subunit hCNRA2

Using the plasmid pUC19BgI/BglII-EcoRI/hCNRA2 as a PCR template, one copy of the autoinhibitory domain (Hubbard *et al.*, Biochemistry (1989), **28**, 1868-1874 and Hashimoto *et al.*, J. Biol. Chem. (1990), **165**, 1924-1927) from hCNRA2 is isolated as a BglII-XbaI fragment (the PCR primers are described in SEQ ID NOs 34 and 35). The ACE1C-h(AID) fusion and the expression plasmid (based on pDP34; see Example 6) is constructed, as described in Example 6. The plasmid is named pCS102.

The second copy of h(AID) is isolated as a SpeI-SacI fragment using the primers with SEQ ID NOs 36 and 12 and pCS102 as template (similar to Example 7). The expression plasmid for ACE1C-h(AID)-$(Gly)_4$-h(AID) in pDP34 is constructed in an identical manner to that of pCS101 (see Example 7). The plasmid is named pCS103.

Example 10: Construction of pRH1, the vector for expression of fusion genes between the DNA-binding domain of ACE1 (i.e. ACE1N) and a gene of interest

The plasmid pTM9 (Munder *et al.*, Mol. Cell. Biol. (1992), **12**, 2091-2099) is digested with EcoRI and KpnI. The ~4400 bp fragment is isolated on a 1% agarose gel and purified by GeneClean®. A ~300 bp EcoRI-KpnI fragment from pUC19/EcoRI-KpnI/SUC2t (see Example 4) is ligated to the ~4400 bp linearized fragment and transformed in *E. coli*. Analysis of transformants with EcoRI and KpnI yielded a plasmid where the CYC1 terminator in pTM9 is replaced by the SUC2 terminator fragment. This plasmid is named pRH1 (Fig. 2) and is a vector which can be used for integration of any gene which is fused to ACE1N (the DNA-binding domain of the yeast transcription factor ACE1) into the yeast chromosome.

Example 11: Construction of yeast expression vectors for the expression of ACE1N-yCNA1-PHO5t, ACE1N-yCNA2-PHO5t, ACE1N-yCNA1Δ-PHO5t, ACE1N-yCNA2Δ-PHO5t, ACE1N-hCNRA2-SUC2t and ACE1N-hCNRA2Δ-SUC2t in pRH1

The subcloning of ACE1N-yCNA1-PHO5t, ACE1N-yCNA2-PHO5t, ACE1N-yCNA1Δ-PHO5t and ACE1N-yCNA2Δ-PHO5t in pRH1 is performed in an identical manner. A BglII-HindIII fragment containing the complete yCNA1 or yCNA2 genes (or their truncated versions; Examples 1 and 2) and the HindIII-KpnI fragment of the yeast PHO5 transcription terminator are ligated to pRH1 (see Example 10) which is completely digested with BglII and KpnI. Restriction enzyme analysis of DNA, obtained from *E. coli* transformants, reveals the correct clones. The plasmids are named pMH6, pMH7 (encoding ACE1N-yCNA1 and ACE1N-yCNA1Δ, respectively) and pMH21, pMH22 (encoding ACE1N-yCNA2 and ACE1N-yCNA2Δ, respectively)

The BglII-EcoRI fragments containing the hCNRA2 and hCNRA2Δ genes (see Example 8; both lacking the DNA encoding the N-terminal residues, Met^1, Ala^2) are subcloned directly in BglII-EcoRI digested pRH1. The plasmids are named pKO11, pKO12 (encoding the ACE1N-hCNRA2 and ACE1N-hCNRA2Δ fusions, respectively).

Example 12: Construction of a yeast vector for expression of yTPK1, the complete gene encoding the catalytic subunit of yeast cAMP-dependent protein kinase

A ~1200 bp BglII-EcoRI fragment of the yTPK1 gene (the complete coding sequence; Toda *et al.*, Cell (1987), 50, 277-287) is isolated from the yeast genome by PCR using the primers with SEQ ID NOs 37 and 38. For the expression of yTPK1 as a ACE1N-yTPK1 fusion, the ~1200 bp BglII-EcoRI fragment of yTPK1 is subcloned in pRH1 (see Example 10). One clone, which contains the correct insert (confirmed by restriction enzyme analysis and DNA sequencing) is named pSK1A.

Example 13: Construction of a yeast expression vector for expression of the regulatory subunit of yTPK1, i.e. yBCY1: the complete gene which also encodes the inhibitory peptide of yTPK1

A ~1260 bp BamHI-EcoRI fragment of the yeast BCY1 gene (the complete coding sequence, Toda *et al.*, Mol. Cell. Biol. (1987), 7, 1371-1377) is isolated from the yeast genome by PCR, using the primers with SEQ ID NOs 39 and 40. For the expression of yBCY1 as a ACE1C-yBCY1 fusion, the ~1075 bp SalI-BglII fragment from pRH10C (see Example 5), the ~1260 bp BamHI-EcoRI fragment of the yeast BCY1 gene and the ~300 bp EcoRI-SacI fragment from pUC19/EcoRI-SacI/SUC2t (see Example 4) are ligated to the vector pDP34 which has been completely digested with SalI and SacI. One clone, which contains the correct insert (confirmed by restriction enzyme analysis and DNA sequencing) is named pSK5.

Example 14: Construction of yeast expression vectors for the expression of ACE1C-BCY1(wt ID) ACE1C-BCY1(Mut1 ID) and ACE1C-BCY1(Mut2 ID) which encode a single copy of the gene fragments encoding the inhibitory peptide (ID) of yeast BCY1 (wild type, pseudosubstrate and a mutated pseudosubstrate)

The ~1075 bp SalI-BglII fragment from pRH10C (see Example 5) and double-stranded linkers (BglII-XbaI fragments) encoding BCY1(wt-ID) (SEQ ID NO 41), BCY1(Mut1-ID) (SEQ ID NO 42) and BCY1(Mut2-ID) (SEQ ID NO 43) are ligated to pUC19 completely digested with SalI and XbaI. The resulting correct clones are named pUC19/SalI-XbaI/GAPCLp-ACE1C-BCY1(wt-ID), pUC19/SalI-XbaI/GAPCLp-ACE1C-BCY1(Mut1-ID) and pUC19/SalI-XbaI/GAPCLp-ACE1C-BCY1(Mut2-ID).

The individual SalI-XbaI fragments from the above plasmids and a XbaI-SacI fragment from pUC19/XbaI-SacI/SUC2t (Example 4) are subcloned in pDP34 (as described in Example 6). The expression plasmids are named pSK26, pSK21 and pSK22 [encoding ACE1C-BCY1(wt-ID)], pSK21 [encoding ACE1C-BCY1(Mut1-ID)] and pSK22 [encoding ACE1C-BCY1(Mut2-ID)].

Example 15: Construction of yeast expression vectors for the expression of ACE1C-BCY1(Mut1 ID)-$(Gly)_4$-BCY1(Mut1 ID) and ACE1C-BCY1(Mut2 ID)-$(Gly)_4$-

BCY1(Mut2 ID), the gene fragments encoding the inhibitory peptides of yeast BCY1 (pseudosubstrate and a mutated pseudosubstrate): two copies linked by glycine linkers

The second copies of BCY1(wt-ID), BCY1(Mut1-ID) and BCY1(Mut2-ID) are isolated by PCR using the primers (SEQ ID NOs 44 and 45), the templates being pSK26, pSK21 and pSK22, respectively. Expression plasmids in pDP34 are made as described in Example 7.

Example 16: Isolation of the three fragments from human p70 s6 kinase (p70s6k), the complete gene, a C-terminal truncated version of p70s6k (p70s6kΔC) and an N-terminal truncated version of p70s6k (p70s6kΔN), for expression as fusion genes with ACE1N

In order to obtain the genes encoding p70s6k, p70s6kΔC and p70s6kΔN, for correct in-frame fusion with the DNA-binding domain of the yeast transcription activator ACE1, PCR is performed on the cDNA encoding the mitogen-activated S6 kinase (i.e. p70s6k) obtained from the rat liver (Kozma *et al.*, Proc. Natl. Acad. Sci. USA (1990), **87**, 7365-7369). The rat gene encodes a polypeptide identical to the human protein. Three pairs of deoxyoligoribonucleotides are used as primers (see SEQ ID NOs 45 and 46; 45 and 47 and 48 and 46).

The genes, obtained as BamHI-XbaI/ BamHI-EcoRI fragments, are subcloned in pRH1 (see Example 10). The resulting plasmids are named pSK18 (encoding wild type (wt) p70s6k), pSK6 (encoding p70s6kΔC) and pSK27 (encoding p70s6kΔN), respectively.

Example 17: Construction of yeast expression vectors for the expression of ACE1C-s6k(wt AID), ACE1C-s6k(Mut1-AID) and ACE1C-s6k(Mut2-AID): single copies of the gene fragments encoding the autoinhibitory peptides of human p70 s6 kinase (wild type, pseudosubstrate and a mutated pseudosubstrate)

The BglII-EcoRI ~312 bp fragments, containing the wild type or mutated autoinhibitory peptide sequences (Banerjee, *et al.*, Proc. Natl. Acad. Sci. USA (1990), **87**, 8550-8554) are subcloned in the context of the activation domain of the yeast transcriptional activator ACE1 in the expression vector pDP34 (as in Example 6). The pair of deoxyoligoribonucleotides with SEQ ID NOs 49 and 46 are used to isolate the wild type (wt) and the two mutated

sequences as BglII-EcoRI fragments. The resulting plasmids are named pSK11 (wt), pSK13 (Mut1) and pSK12 (Mut2).

The four Ser/Thr residues comprising the autophosphorylation sites in the autoinhibitory peptide of p70s6k (Ferrari *et al.*, Proc. Natl. Acad. Sci. USA (1992), **89**, 7282-7286) are mutated to Ala in the Mut1-AID sequence (Ferrari *et al.*, J. Biol. Chem. (1993), **268**, 16091-16094), whereas in Mut2-AID the Ser/Thr residues are replaced by the acidic amino acids Glu/ Asp (Flotow *et al.*, J. Biol. Chem. (1992), **267**, 3074-3078).

A BglII-EcoRI ~513 bp fragment (comprising the above ~312 bp fragment and a 5' extension of ~200 bp) is subcloned by PCR from the wt gene and the mutated sequences, which encode the Mut1-AID and Mut2-AID mutations. The pair of deoxyoligoribonucleotides with SEQ ID NOs 50 and 46 are used to isolate the wt and the two mutated sequences as BglII-EcoRI fragments. They are cloned as ACE1C fusions in pDP34, as detailed in Example 6. They are referred to as pSK8 (wt), pSK10 (Mut1) and pSK9 (Mut2).

Example 18: Construction of plasmids for the expression of p70s6k, p70s6kΔC and p70s6kΔN from yeast multi-copy vectors

A ~670 bp SalI-BglII fragment (containing the GAPCL promoter) is isolated from pRH2 (see Example 5) by PCR using the primers with the SEQ ID NOs 51 and 52.

The above SalI-BglII fragment, the BamHI-XbaI/ BamHI-EcoRI fragments (encoding the sequences of p70s6k, p70s6kΔC and p70s6kΔN; see Example 16) and a XbaI-SacI/ EcoRI-SacI fragment of the SUC2 terminator (see Example 4) are subcloned in pDP34 digested completely with SalI and SacI. The correct clones are named pSK16 (encoding p70s6k), pSK18 (encoding p70s6kΔC) and pSK28 (encoding p70s6kΔN).

It is observed that yeast transformants of pSK16 yield an active protein. No protein is seen in cells harboring pSK18 and pSK28. However, the amount of mRNA obtained from the latter is the same as in cells expressing full length p70S6k.

Example 19: Chromosomal integration of ACE1N fusion genes in the yeast strain TFY2

The Bio-Rad gene pulser is employed for transformation of yeast cells (see Table 1) by electroporation (Methods. Enzymol. (1991), 194, 182-187). The strain TFY2 (Matα his ura3-52 trp1-285 ace1 LEU2::YipCL CUP1) (Munder *et al.*, Mol. Cell. Biol. (1992), 12, 2091-2099) is used for all integrations of ACE1N-gene fusions into the yeast chromosome. Correct gene integration and gene replacement events are verified by PCR with primers flanking the insertion sites. Subsequently, the amplified fragments are analyzed by agarose gel electrophoresis.

TABLE1: (Yeast strains used)

Fusion	Strain	Genotype
-	TFY2	Matα his ura3-52 trp1-285 ace1 LEU2::YipCL CUP1^r
ACE1N-yCNA1	CNY1	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pMH6
ACE1N-yCNA1Δ	CNY2	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pMH7
ACE1N-yCNA2	CNY3	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pMH21
ACE1N-yCNA2Δ	CNY4	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pMH22
ACE1N-hCNRA2	CNY5	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pKO11
ACE1N-hCNRA2Δ	CNY6	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pKO12
ACE1N-yTPK1	CNY7	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pSK1A
ACE1N-yp70s6k	CNY8	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pSK18
ACE1N-yp70s6kΔC	CNY9	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pSK6
AC0E1N-yp70s6kΔN	CNY10	Matα his ura3-52 ace1 LEU2::YipCL CUP1^{r}TRP1::pSK27

Example 20: Yeast transformations with plasmids, in strains harboring gene fusions with ACE1N

Yeast strains harboring ACE1N gene fusions are transformed with plasmids bearing the different ACE1C fusions as described in Example 19. The transformants are depicted in Table 2.

TABLE 2: plasmids and the corresponding strains used for creating protein-interactions

Plasmid	ACE1C fusion	Yeast strain	Yeast transformant	Growth in $CuSO_4^r$	β-gal activity
pMH10	y1(AID)	CNY1	CNY11	+	
pMH11	y1(AID)-Gly_4-y1(AID)	CNY1	CNY12	++	++
pRH14C	-	CNY1	CNY13	-	-
pMH10	y1(AID)	CNY2	CNY14	nd	nd
pMH11	y1(AID)-Gly_4-y1(AID)	CNY2	CNY15	nd	nd
pRH14C	-	CNY2	CNY16	nd	nd
pCS100	y2(AID)	CNY3	CNY17	nd	nd
pCS101	y2(AID)-Gly_4-y2(AID)	CNY3	CNY18	nd	nd
pRH14C	-	CNY3	CNY19	nd	nd
pCS100	y2(AID)	CNY4	CNY20	nd	nd
pCS101	y2(AID)-Gly_4-y2(AID)	CNY4	CNY21	nd	nd
pRH14C	-	CNY4	CNY22	nd	nd
pCS102	h(AID)	CNY5	CNY23	nd	nd
pCS103	h(AID)-Gly_4-h(AID)	CNY5	CNY24	nd	nd
pRH14C	-	CNY5	CNY25	nd	nd
pCS101	h(AID)	CNY6	CNY26	nd	nd
pCS103	h(AID)-Gly_4-h(AID)	CNY6	CNY27	nd	nd
pRH14C	-	CNY6	CNY28	nd	nd
pSK5	BCY1	CNY7	CNY29	++	++
pSK26	BCY1(wt-ID)	CNY7	CNY30	+/-	+/-
pSK21	BCY1(Mut1-ID)	CNY7	CNY31	+	+
pSK23	BCY1(Mut1-ID)-Gly_4-(Mut1-ID)	CNY7	CNY32	++	++
pSK22	BCY1(Mut2-ID)	CNY7	CNY33	-	-
pSK24	BCY1(Mut2-ID)-Gly_4-(Mut2-ID)	CNY7	CNY34	-	-
pSK11	312bp wt(AID)	CNY8	CNY35	+	+

pSK13	312bp Mut1(AID)	CNY8	CNY36	++	++
pSK12	312bp Mut2(AID)	CNY8	CNY37	-	-
pSK8	513bp wt(AID)	CNY8	CNY38	+	+
pSK10	513bp Mut1(AID)	CNY8	CNY39	++	++
pSK9	513bp Mut2(AID)	CNY8	CNY40	-	-
pSK11	312bp wt(AID)	CNY9	CNY41	-	-
pSK13	312bp Mut1(AID)	CNY9	CNY42	-	-
pSK12	312bp Mut2(AID)	CNY9	CNY43	-	-
pSK8	513bp wt(AID)	CNY9	CNY44	-	-
pSK10	513bp Mut1(AID)	CNY9	CNY45	-	-
pSK9	513bp Mut2(AID)	CNY9	CNY46	-	-
pSK11	312bp wt(AID)	CNY10	CNY47	-	-
pSK13	312bp Mut1(AID)	CNY10	CNY48	-	-
pSK12	312bp Mut2(AID)	CNY10	CNY49	-	-
pSK8	513bp wt(AID)	CNY10	CNY50	-	-
pSK10	513bp Mut1(AID)	CNY10	CNY51	-	-
pSK9	513bp Mut2(AID)	CNY10	CNY52	-	-

nd = not determined

Example 21: Assay for copper resistance: induction of metallothionein expression

Equal numbers of cells are spotted onto SD-agar plates (2 % glucose, 0.67 % yeast nitrogen base without amino acids, 2 % agar) containing (a) no $CuSO_4$, (b) 100 µM $CuSO_4$ and (c) 200 µM $CuSO_4$ (Munder *et al.*, Mol. Cell. Biol. (1992), 12, 2091-2099). The results are portrayed in Table 2.

Example 22: β-galactosidase assay

Yeast cells (Example 19) are grown in YPD medium (1 % bacto-yeast extract, 2 % bacto-peptone, 2 % glucose) for 17 h at 30°C. Cells are first washed in SD medium (0.67 % yeast nitrogen base without amino acids, 2 % glucose) and then the transcription of the reporter

gene is induced. Induction and permeabilization of cells are performed as described previously (Fürst *et al.*, Cell (1988), 55, 705-717). β-galactosidase activity, measured as o-nitrophenyl-β-D-galacto-pyranoside hydrolysis at 420 nm, is normalized to cell culture density and expressed as arbitrary units. The activity in the control strains, harboring the plasmid pRH14C, is set to the value 1. The results are portrayed in Table 2.

Example 23: Disruption of protein-peptide interactions with small organic compounds

Cells are initially grown in YPD, as in Example 22. After washing in SD, cells are preincubated with small organic compounds (dissolved in dimethyl sulphoxide and 10-20 μmolar in final concentration) for 90 min at 30°C. Later, β-galactosidase activity is measured, exactly as in Example 22.

Example 24: Disruption of protein-peptide interaction in CNY15 with the immunosuppressants cyclosporin A and FK506

Three individual transformants from CNY15 (see Table 2) are grown in YPD, as in Example 22. In the strains, interaction between y1CNA1D (yeast calcineurin isozyme 1 from which the C-terminal autoinhibitory domain is deleted) and y1(AID)-Gly_4-y1(AID) [the autoinhibitory domain of the same enzyme; two copies linked by 4 glycines] are established. After washing in SD, cells are preincubated with cyclosporin A (CsA; final concentrations: 0, 5 and 10 μg/ ml) and FK506 (final concentrations: 0, 0.5 and 1 μg/ ml) for 15 h at 30°C. Later, β-galactosidase activity is measured, as in Example 22. As control, three individual transformants from CNY16 (see Table 2) are used. The results, an average of three transformants (in duplicate), are depicted in Table 3. The units shown are arbitrary (see Example 22).

TABLE 3. Disruption of the protein-peptide interaction in CNY15

Strain	Compound	Final concentration in µg/ ml	Relative β-Galactosidase units
CNY15	CsA	0	3
CNY15	CsA	5	1.8
CNY15	CsA	10	1
CNY15	FK506	0	3
CNY15	FK506	0.5	1.6
CNY15	FK506	1	1
CNY16	CsA/ FK506	0	1
CNY16	CsA/ FK506	5/0.5	0.95
CNY16	CsA/ FK506	10/1	0.92

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CIBA-GEIGY AG
(B) STREET: Klybeckstr. 141
(C) CITY: Basel
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4002
(G) TELEPHONE: +41 61 69 11 11
(H) TELEFAX: + 41 61 696 79 76
(I) TELEX: 962 991

(ii) TITLE OF INVENTION: Dual hybrid system

(iii) NUMBER OF SEQUENCES: 52

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..28
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of yest CNA1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATAGATCTAA TGTCGAAAGA CTTGAATT 28

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..30)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end anti-sense strand of conding
sequence of yeast CNA1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATAAGCTTTC ACAGTTGTGG CTTTTTCTCC 30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "fragment of yeast CNA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATAGATCTAA TGTCTTCAGA CGCTATAAG 29

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindIII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..30)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end anti-sense strand of coding sequence of CNA2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATAAGCTTCT ATTTGCTATC ATTCTTTGCA 30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindIII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..30)

(D) OTHER INFORMATION: /function= "PCR primer"
/note= "5' anti-sense strand of coding sequence of CNA1 delta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATAAGCTTCT ACAAACCTTC AGTCCCACGA 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindIII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..31)
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "5' end anti-sense strand of coding sequence of CNA2 delta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATAAGCTTCT ATAAACCCTT TACACCATTA G 31

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..28
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "fragment of y1(AID) from CNA1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATAGATCTGG GTTTGAATGA AACGCTAA 28

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8

(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..26)
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "5' end anti-sense strand of coding sequence of y1(AID) from CNA1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TATCTAGACT TTAATATTTT TTCGTA 26

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "fragment of y2(AID) from CNA2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATAGATCTGG GTTTAGATGA AGCCCTGTC 29

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end anti-sense strand of coding sequence of y2(AID)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATTCTAGATT TCTGCCAAAC TTTTTCGT 28

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(ix) FEATURE:
(A) NAME/KEY: terminator
(B) LOCATION: 9..28
(D) OTHER INFORMATION: /standard_name= "SUC2 terminator"
/note= "fragment for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATGAATTCAG GTTATAAAAC TTATTGTC 28

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SacI site"

(ix) FEATURE:
(A) NAME/KEY: terminator
(B) LOCATION: complement (9..26)
(D) OTHER INFORMATION: /standard_name= "SUC2 terminator"

/note= "Anti-sense strand fragment for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TAGAGCTCGG TCCATCCTAG TAGTGT 26

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: terminator
(B) LOCATION: 9..28
(D) OTHER INFORMATION: /standard_name= "SUC2 terminator"
/note= "fragment for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATTCTAGAAG GTTATAAAAC TTATTGTC 28

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII"

(ix) FEATURE:
(A) NAME/KEY: terminator
(B) LOCATION: 9..28
(D) OTHER INFORMATION: /standard_name= "SUC2 terminator"
/note= "fragment for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATAGATCTAG GTTATAAAAC TTATTGTC 28

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "KpnI site"

(ix) FEATURE:

(A) NAME/KEY: terminator
(B) LOCATION: complement (9..26)
(D) OTHER INFORMATION: /standard_name= "SUC2 terminator"
/note= "anti-sense fragment for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TAGGTACCGG TCCATCCTAG TAGTGT 26

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..4
(D) OTHER INFORMATION: /function= "EcoR1 site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (75..78)
(D) OTHER INFORMATION: /function= "SpeI site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 5..74
(D) OTHER INFORMATION: /note= "Sense and anit-sense strand of nuclear localization signal of SV40 T antigene using yeast codons"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AATTCATGGA CAAGGTCTTC AGAAACTCTT CCAGAACTCC ACCAAAGAAG AAGAGAAAGG 60

TTGAAGACCC AGCACTAG 78

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..26
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "yeast ACE1 transcription activation domain fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TATCTAGAGG ACGTTCTTTT GGGCCT 26

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (11..29)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' anti-sense strand of ACE1 transcription activation domain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TAAGATCTGC TTGTGAATGT GAGTTATGC 29

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..4)
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 19..22
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 6..17
(D) OTHER INFORMATION: /function= "Glycine linker"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

22

CTAGTGGTGG CGGTGGCTGA TC

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SpeI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 9..27
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "sense strand of y1(AID) (second copy) from yeast CNA1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATACTAGTGG TTTGAATGAA ACGCTAA 27

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindIII"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..30)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' anti-sense strand of coding sequence of
y1(AID) (for second copy) from yeast CNA1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ATAAGCTTTC ACTTTAATAT TTTTTCGTAG 30

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SpeI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 9..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of y2(AID) (second copy) from CNA2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

ATACTAGTGG ATTCTCTCCA CCACATAGA 29

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 3..24
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of human CNRA2 gene"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 11..16
(D) OTHER INFORMATION: /function= "NcoI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GTGGTGACAT CCATGGCCAA TTTT 24

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (1..25)
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "anti-sense fragment of coding sequence of human CNRA2 gene"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SacI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

ATGAGCTCTA ATAATCGATA ACAAA 25

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..25
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of human CNRA2 gene"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 5..10
(D) OTHER INFORMATION: /function= "NcoI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

ATTAGAGCTC ATGAAGCTCA AGATG 25

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..47)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "anti-sense strand of sequence coding for human CNRA2 gene"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ATGAATTCTCA CTGGGCACTA TGGTTGCCAG TCCCGTGGTT CTCAGT 47

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 165 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..165
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "coding for first 55 amino acids of hCNRA2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

ATGGCTGCTC CAGAACCAGC TAGAGCTGCA CCACCACCAC CTCCACCACC TCCACCACCT 60

CCAGGTGCTG ACAGAGTCGT CAAAGCTGTC CCTTTCCCAC CAACACATCG CTTGACATCT 120

GAAGAAGTAT TTGATTTGGA TGGGATACCC AGGGTTGATG TTCTG 165

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 165 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 9..161
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "anti-sense strand of of coding sequence of human CNR2 gene (yeast-biased codons)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCATGGATGT CACCACACAC TGTGATTGGA GCTTCTACTT CTATCATGGT TTTCTCTCTC 60

CGAAGGATGG CAGCACCCTC ATTGATAATT CTAAGCGCAA TTTCTTCATC TACTCGACCT 120

TCTTTCACCA AGTGGTTCTT CAGAACATCA ACCCTGGGTA TCCCA 165

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of human hCNRA2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

ATAGATCTCA TGGCTGCTCC AGAACCAGC 29

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (2..24)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "anti-sense strand of human CNRA2"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6..11

(D) OTHER INFORMATION: /function= "NcoI site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

ATTGGCCATG GATGTCACCA CACA 24

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR primer"
/note= "fragment of human CNRA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

ATAGATCTTG CTCCAGAACC AGCTAGAGC 29

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end of anti-sense strand of human CNRA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

ATGAATTCTC ACTGGGCAGT ATGGTTGC 28

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..29)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end of anti-sense strand of human CNRA2 delta"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

ATGAATTCTC ATATTGCTTT TTCAGCCTC 29

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of h(AID) from human CNRA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ATAGATCTAG GATTCTCTCC ACCACATAG 29

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..29)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end of anti-sense strand of coding sequence of y2(AID) from human CNA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

ATTCTAGAAT CTTGCTGTAC AGCATCTTT 29

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SpeI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 9..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of h(AID), (second copy) from human CNRA2 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

ATACTAGTGG ATTCTCTCCA CCACATAGA 29

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..30
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of yeast TPK1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

ATAGATCTTA TGTCGACTGA AGAACAAAAT 30

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "HindIII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end of antisens strand of coding sequence of yeast TPK1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

ATGAATTCTT AGAAGTCCCG GAAAAGAT 28

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BamHI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "fragment of yeast BCY1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

ATGGATCCGA TGGTATCTTC TTTGCCCAA 29

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(ix) FEATURE:

(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' end of anti-sense strand of coding sequence of yeast BCY1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

ATGAATTCTT AATGTCTTGT AGGATCAT 28

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..5
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (67..71)
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 6..66
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "BCY1(wt-ID) from yeast BCY1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

GATCTGACAT CAACTCCTCC ACTCCCAATG CACTTCAACG CCCAAAGGCG TAATGCTGTT 60

AGTGGTTCTA G 71

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..5
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (68..71)
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 6..67
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "BCY1(Mut1-ID) from yeast BCY1 gene.
Nucleotides 53 and 55 are changed to replace
ThrSer by AsnAla."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GATCTGACAT CAACTCCTCC ACTCCCAATG CACTTCAACG CCCAAAGGCG TAATGCTGTT 60

AGTGGTTCTA G 71

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..5
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (68..71)
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 6..67
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "BCY1(Mut2-ID) from yeast BCY1 gene.
Nucleotides 46, 47, 49, 51, 53 and 55 are changed
to replace ArgArgThrSer by AlaAlaAsnAla"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GATCTGACAT CAACTCCTCC ACTCCCAATG CACTTCAACG CCCAAGCGGG CAATGCTGTT 60

71

AGTGGTTCTA G

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SpeI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 9..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "BCY1(wt-ID or Mut1-ID or Mut2-ID) (second copy) from yeast BCY1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

29

ATACTAGTAC ATCAACTCCT CCACTCCCA

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BamHI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "rat p70S6 kinase gene fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

ATGGATCCGA TGGCAGGAGT GTTTGACAT 29

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "XbaI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"

/note= "5'end anti-sense strand of coding sequence of rat p70S6 kinase gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

ATTCTAGAGA AATCCCCAGG AGAGAATT 28

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "EcoRI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5'end anti-sense strand of coding sequence of truncated p70S6k delta C gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

ATGAATTCTC ATAGATTCAT ACGCAGGT 28

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BamHI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..30
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "truncated p70S6k delta N gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

ATGGATCCAG AATGTTTTGA GCTACTTCGG 30

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..32
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "sense strand of the ~312 bp autoinhibitory sequence from p70S6K gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

ATAGATCTGA AAGAAAAGTT TTCTTTTGAA CC 32

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..29
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "sense strand of ~513 bp autoinhibitory sequence from p70S6k gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

ATAGATCTTA ACTGGGAAGA GCTTTTGGC 29

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "SalI site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 10..28
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "sense strand of ~650 bp pBR322 (~275 bp)
and the GAPCL (~396 bp) promoter fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

ATGTCGACGC TCTCCCTTAT GCGACTCC 28

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3..8
(D) OTHER INFORMATION: /function= "BglII site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: complement (9..28)
(D) OTHER INFORMATION: /function= "PCR fragment"
/note= "5' anti-sense strand of the GAPCL (~396 bp) promoter fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

ATAGATCTTT TGTTTATGTG TGTTTATT 28

Claims

1. A transcription system for measuring the interaction between a phosphatase or kinase, or a mutein or fragment thereof that binds an autoinhibitory domain; with said autoinhibitory domain, comprising
   a) the DNA binding domain of a transcription factor and the transcription activation domain of a transcription factor that are separated, wherein one of the two transcription factor domains is linked to a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain; and the other of the two transcription factor domains is linked to a polypeptide comprising an autoinhibitory domain that is capable of binding to the polypeptide linked to the first transcription factor domain, and
   b) a DNA that is transcribed when the DNA binding domain of said transcription factor and the transcription activation domain of said transcription factor are connected *via* the polypeptides linked thereto.

2. A transcription system according to claim 1, wherein genuine phosphatase or kinase comprises an autoinhibitory domain.

3. A transcription system according to claim 1, wherein the autoinhibitory domain and the fragment that binds an autoinhibitory domain originate from the same enzyme komplex.

4. A transcription system according to claim 1, wherein the autoinhibitory domain and the fragment that binds an autoinhibitory domain originate from the same enzyme.

5. A transcription system according to claim 1, wherein said phosphatase or kinase or the mutein or fragment of said phosphatase or kinase has a free AID binding domain.

6. A phosphatase or kinase according to claim 1, selected from the group consisting of calcineurin, calcineurin that does not comprise the C-terminal 40 to 60 amino acids, PKA and p70S6 kinase.

7. An autoinhibitory domain according to claim 1, wherein the region that binds to the phosphatase or kinase according to claim1 is present in 1 to 5 copies.

8. An autoinhibitory domain according to claim 7, wherein said region is present in 2 to 4 copies.

9. An autoinhibitory domain according to claim 7, wherein these binding regions are the same or different.

10. An autoinhibitory domain according to claim 7, wherein the binding regions are connected directly or via spacer groups.

11. An autoinhibitory domain according to claim 1, selected from the group consisting the autoinhibitory domain of calcineurin, the regulatory subunit of PKA, and the autoinhibitory domain of p70S6; and fragments thereof that are still capable of binding to the phosphatase or kinase.

12. An autoinhibitory domain according to claim 1, comprising a fragment of calcineurin comprising amino acid 508 to 533 and/or a fragment of p70S6 comprising amino acid 400 to 432.

13. A transcription system according to claim 1, wherein the DNA binding domain of a transcription factor and the transcription activation domain of a transcription factor originate from the same transcription factor.

14. A transcription system according to claim 1, wherein the transcription factor is selected from the group consisting of Ace1, Gal4, VP16, p53 and lexA.

15. A transcription system according to claim 1, wherein the transcription factor is Ace1

16. A DNA binding domain according to claim 1, comprising the N-terminal 122 amino acids of Ace1.

17. A transcription activation domain according to claim 1, comprising the C-terminal 100 amino acid of Ace1.

18. An expression cassette for the expression of a fusion protein comprising the DNA binding domain or the transcription activation domain of a transcription factor according to claim 1 and
a polypeptide comprising a phosphatase or kinase or a fragment thereof that binds an autoinhibitory domain according to claim 1; or
a polypeptide comprising said autoinhibitory domain or an active fragment thereof, according to claim 1.

19. An expression cassette comprising a promoter that is operably linked to a DNA that is transcribed when the DNA binding domain of a transcription factor and the transcription activation domain of a transcription factor are joined *via* the polypeptides linked thereto according to claim 1; and to a sequence containing a transcription termination signal; with the proviso that said expression cassette is not already part of the natural genome of the host.

20. An expression cassette according to claim 19, wherein the promoter is selected from the group consisting of the CUP1 promoter, the Gal1 and the Gal10 promoter.

21. An expression cassette according to claim 19, wherein the promoter is the CUP1 promoter.

22. An expression cassette according to claim 19, wherein the DNA coding for a polypeptide encodes a polypeptide whose amount can be determined easily.

23. An expression cassette according to claim 19, wherein the DNA coding for a polypeptide encodes metallothionein, β-galactosidase or luciferase.

24. An expression cassette according to claim 19, wherein the DNA coding for a polypeptide encodes metallothionein or β-galactosidase.

25. A hybrid vector comprising an expression cassette according to claim 18 or 19.

26.A microbiological host comprising a transcription system according to claim1.

27.A microbiological host according to claim 26, selected from the group consisting of yeasts and bacteria.

28.A microbiological host according to claim 26, characterized in that it is a yeast cell.

29.A microbiological host according to claim 26, characterized in that it is *Saccharomyces cerevisiae*.

30.Method for the identification of a calcineurin agonist and antagonist comprising
a) treating a microbiological host according to claim 26 with a test compound, and
b) determining the amount of transcription activation.

31.Method according to claim 30, wherein the transcription activation is measured *via* the expression of the DNA coding for a protein.

32.Method according to claim 31 wherein the amount of the protein or an effect caused by the produced protein is determined.

33.Use of a transcription system according to claim 1 for the identification of a phosphatase or kinase agonist or antagonist.

34.Use of a transcription system according to claim 1 for the identification of a phosphatase or kinase antagonist.

35.A compound identified with the method according to claim 33 for use in a method of treatment.

36.A compound identified with the method according to claim 33 for use in a method of treatment that is characterized by inhibiting T-cell activation and/or prevention of graft rejection.

37. A pharmaceutical composition comprising one or more of the compounds identified with the method according to claim 33 or a pharmacologically acceptable salt thereof, optionally together with pharmacologically suitable carrier

Fig. 1

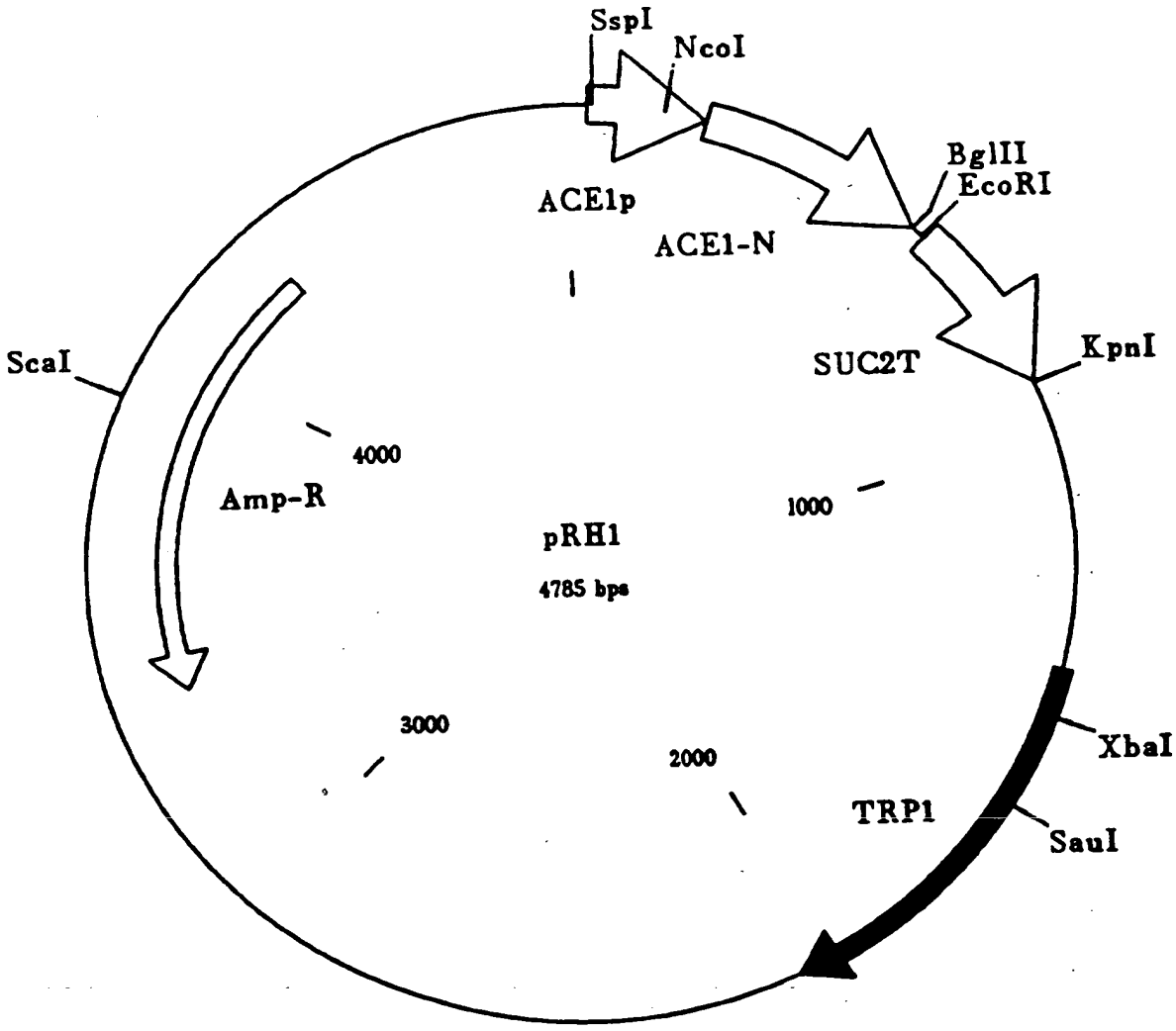
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Fig. 2

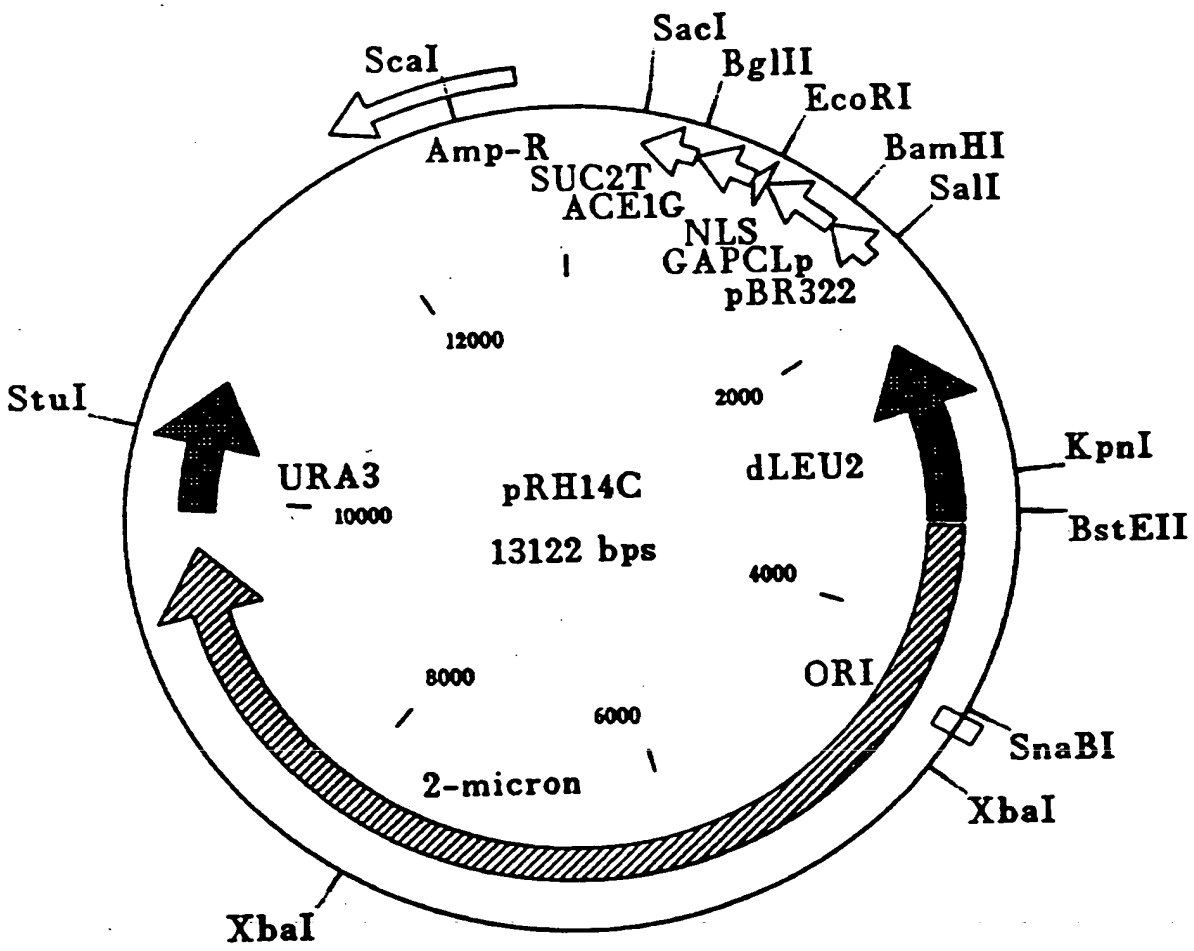
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Fig. 3

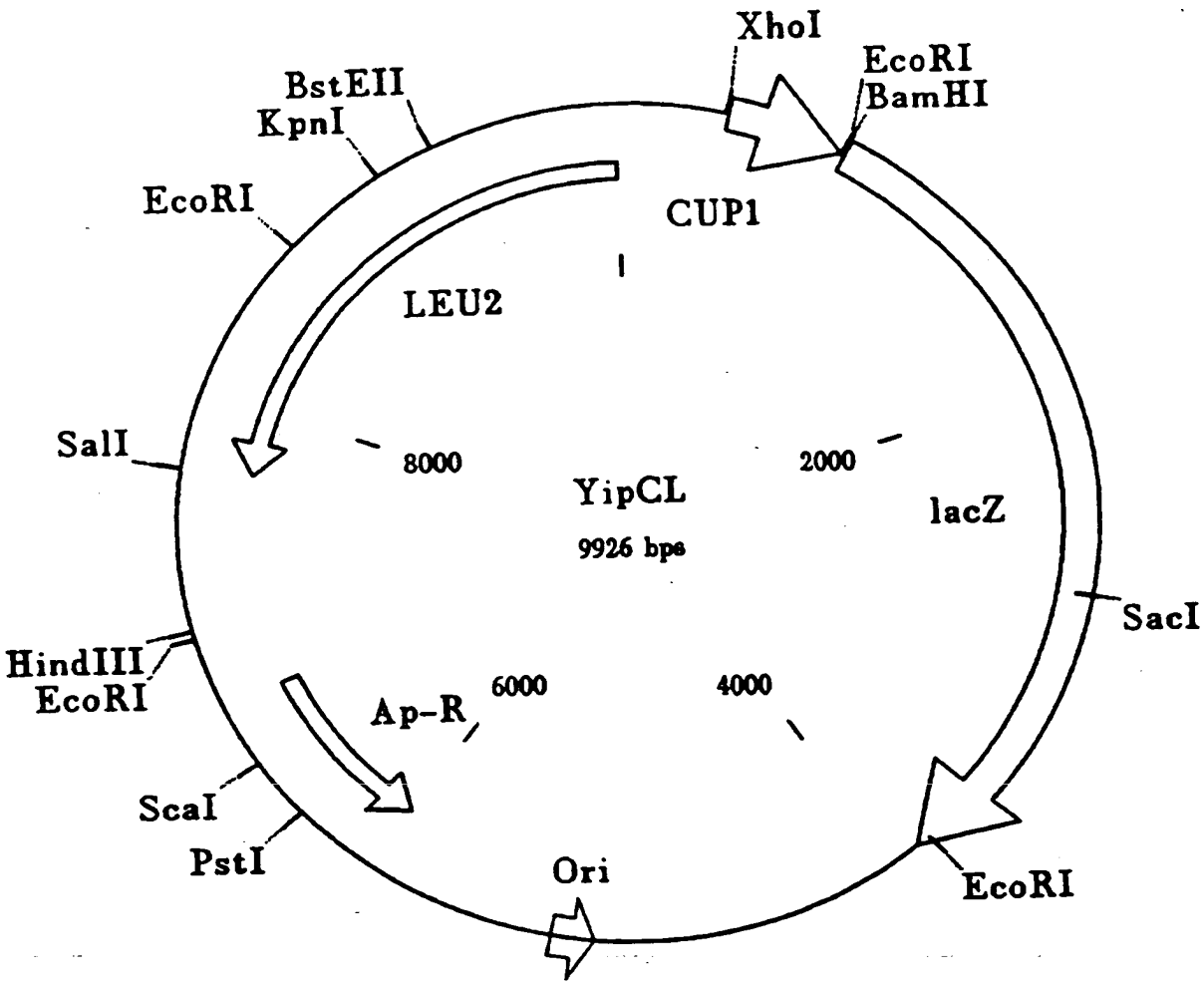
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Fig. 4

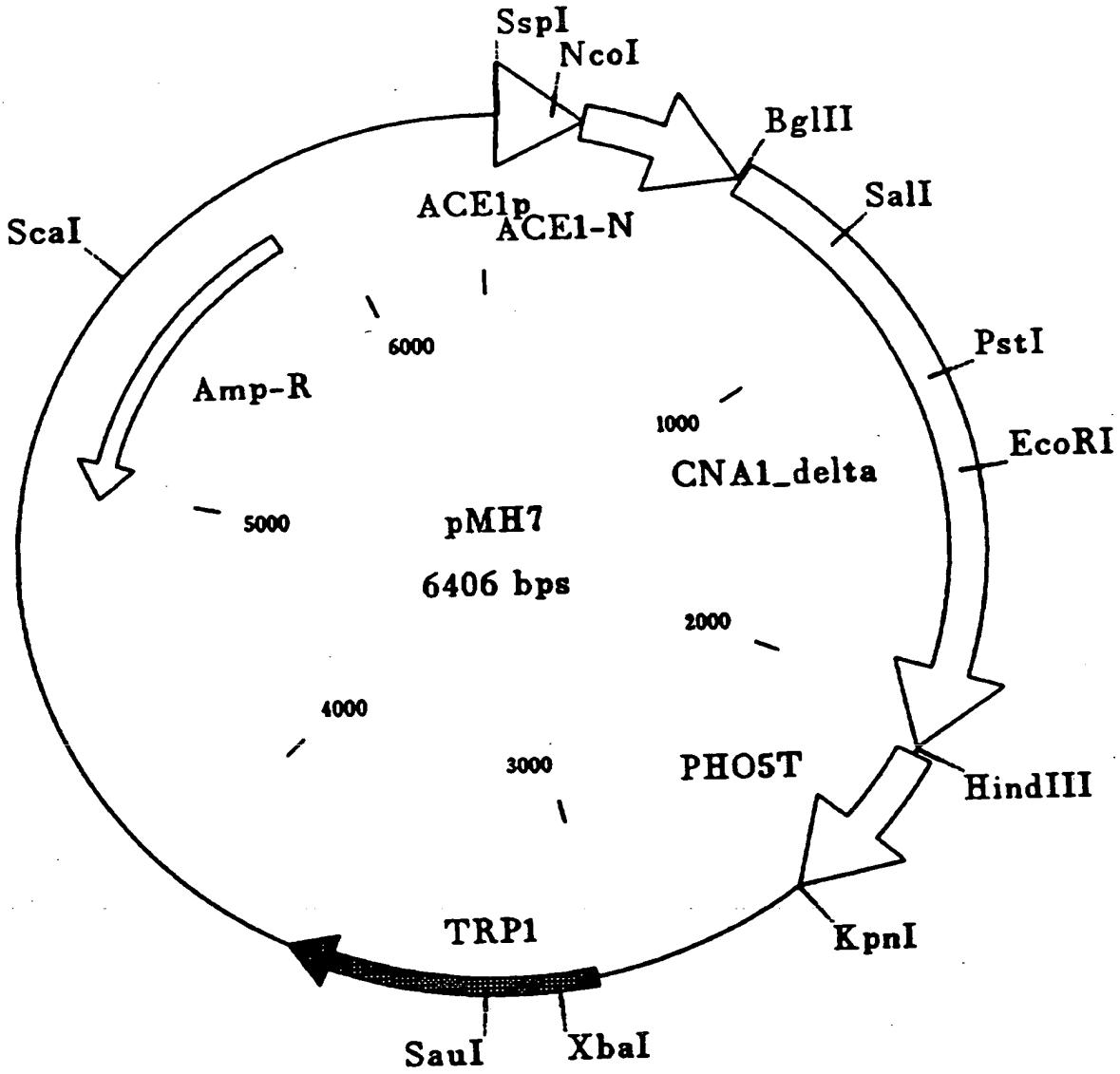
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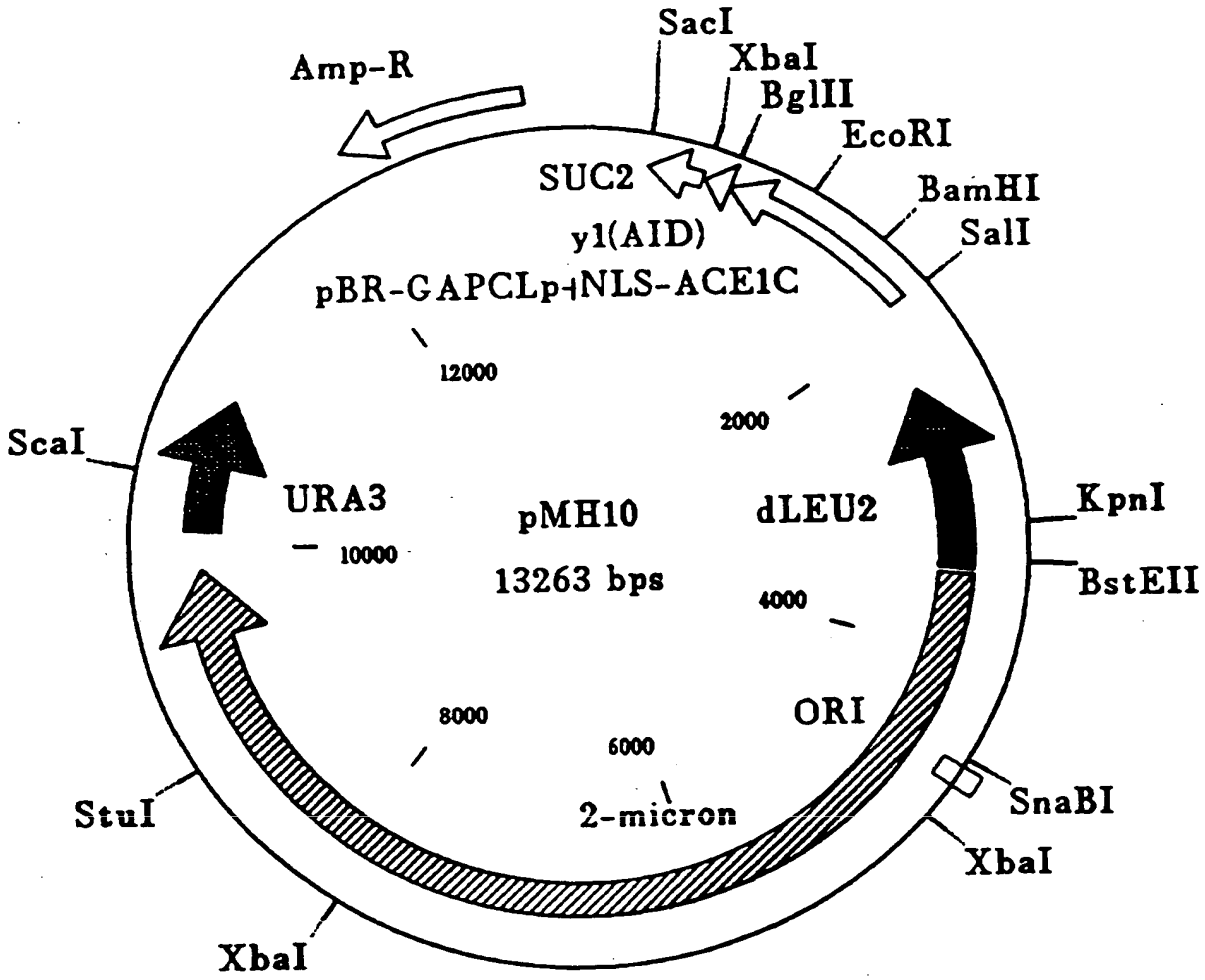
Fig. 5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2-micron
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XbaI
XbaI

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/EP 95/02724

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/81 C12N15/67 C12N15/62 C12Q1/68
C12N1/19 A61K38/17 A61K45/00 //C12N15/54,C12N15/55,
(C12N1/19,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 14780 (KARPAS) 5 August 1993 the whole document ---	35-37
Y	WO,A,94 10300 (THE GENERAL HOSPITAL CORPORATION) 11 May 1994 see page 11, line 19 - page 19, line 22 ---	1-6,11, 13,14, 18-20, 22-34
Y	WO,A,94 09135 (COLD SPRING HARBOR LABORATORY) 28 April 1994 see page 19, line 17 - page 28, line 11 --- -/--	1-6,11, 13,14, 18-20, 22-34

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 1995	17. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A,5 283 173 (FIELDS ET AL.) 1 February 1994 see column 3, line 1 - line 21 see column 3, line 63 - line 67 see claims 1-9	1-6,11, 13,14, 18-20, 22-34
Y	NATURE, vol. 366, 16 December 1993 MACMILLAN JOURNALS LTD., LONDON,UK, pages 704-707, M. SERRANO ET AL. 'A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4' the whole document	1-6,11, 13,14, 18-20, 22-34
Y	SCIENCE, vol. 257, 31 July 1992 AAAS, WASHINGTON, DC, US;, pages 680-682, X. YANG ET AL. 'A protein kinase substrate identified by the two-hybrid system' cited in the application see page 680, line 1 - line 7 see page 682, middle column, line 2 - line 9	1-6,11, 13,14, 18-20, 22-34
Y	J. BIOL. CHEM., vol. 265, no. 4, 5 February 1990 AM. SOC. MOL. BIOL., INC.,BALTIMORE, US;, pages 1924-1927, Y. HASHIMOTO ET AL. 'Identification of an autoinhibitory domain in calcineurin' cited in the application see page 1925, right column, line 30 - page 1927, left column, line 9	1-6,11, 13,14, 18-20, 22-34
A	PROC. NATL. ACAD SCI., vol. 88, no. 16, 15 August 1991 NATL. ACAD SCI., WASHINGTON, DC, US;, pages 7376-7380, M.S. CYERT ET AL. 'Yeast has homologs (CNA1 and CNA2 gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase' cited in the application see page 7379, left column, line 10 - page 7380, left column, line 31; figure 1	1-37

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 95/02724

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. BIOL. CHEM., vol. 267, no. 5, 15 February 1992 AM. SOC. MOL. BIOL., INC.,BALTIMORE, US;, pages 3074-3078, H. FLOTOW AND G. THOMAS 'Substrate recognition determinants of the mitogen-activated 70K S6 kinase from rat liver' cited in the application * the whole document * ---	1-37
A	TRENDS IN BIOCHEMICAL SCIENCE, vol. 15, no. 9, September 1990 ELSEVIER SCIENCE, AMSTERDAM, NL;, pages 342-346, B.E. KEMP AND R.B. PEARSON 'Protein kinase recognition sequence motifs' cited in the application * the whole document * ---	1-37
P,X	FEBS LETT. (1995), 357(2), 221-6 CODEN: FEBLAL;ISSN: 0014-5793, 1995 CHAUDHURI, BHABATOSH ET AL 'The interaction between the catalytic A subunit of calcineurin and its autoinhibitory domain, in the yeast two-hybrid system, is disrupted by cyclosporin A and FK506' the whole document -----	1-36

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02724

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9314780	05-08-93	AU-B-	3364293	01-09-93
		CA-A-	2128790	05-08-93
		EP-A-	0624093	17-11-94
WO-A-9410300	11-05-94	EP-A-	0672131	20-09-95
WO-A-9409135	28-04-94	AU-B-	5444094	09-05-94
		CA-A-	2146965	28-04-94
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US-A-5283173	01-02-94	NONE		

Fig. 1

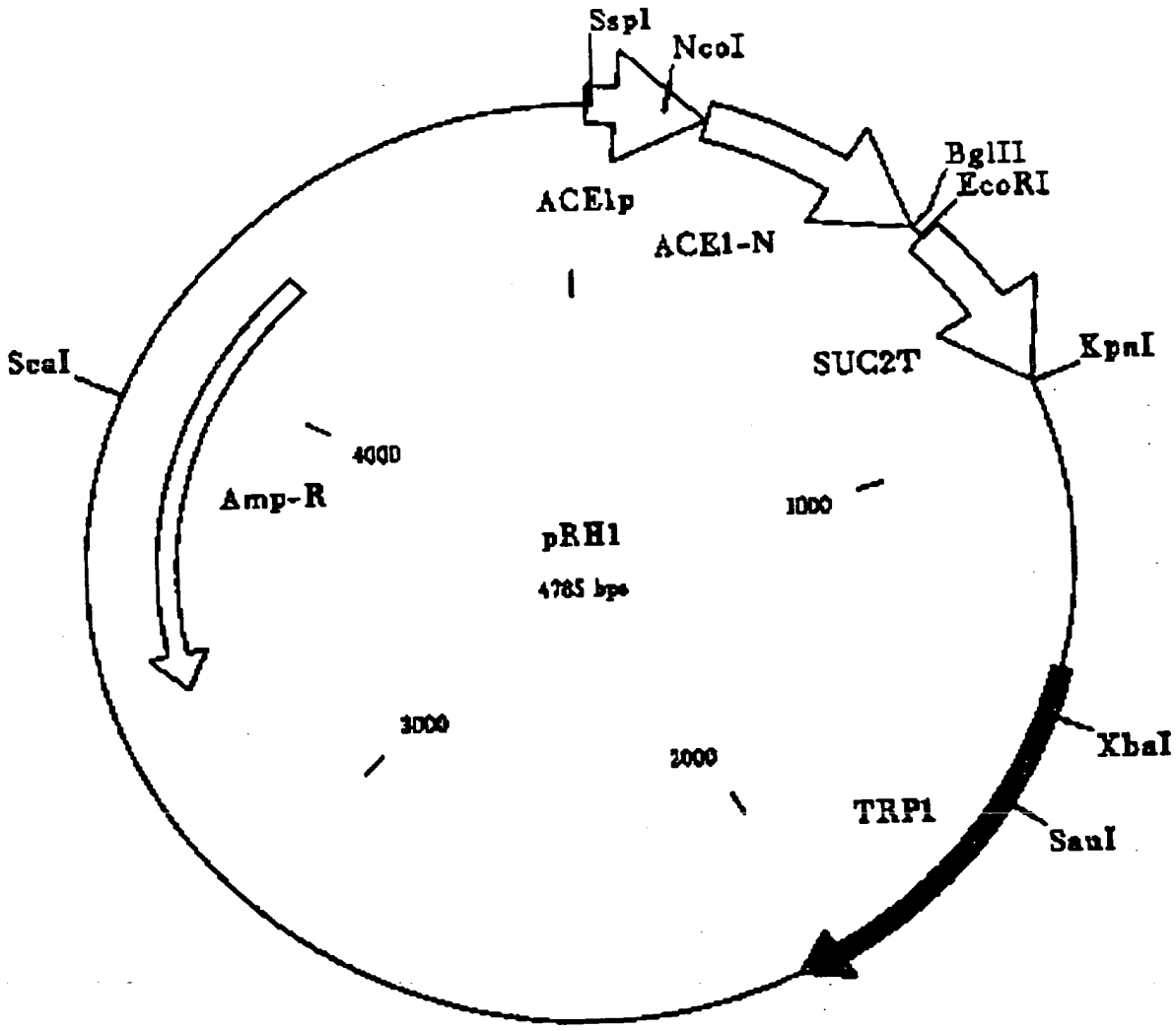
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Fig. 2

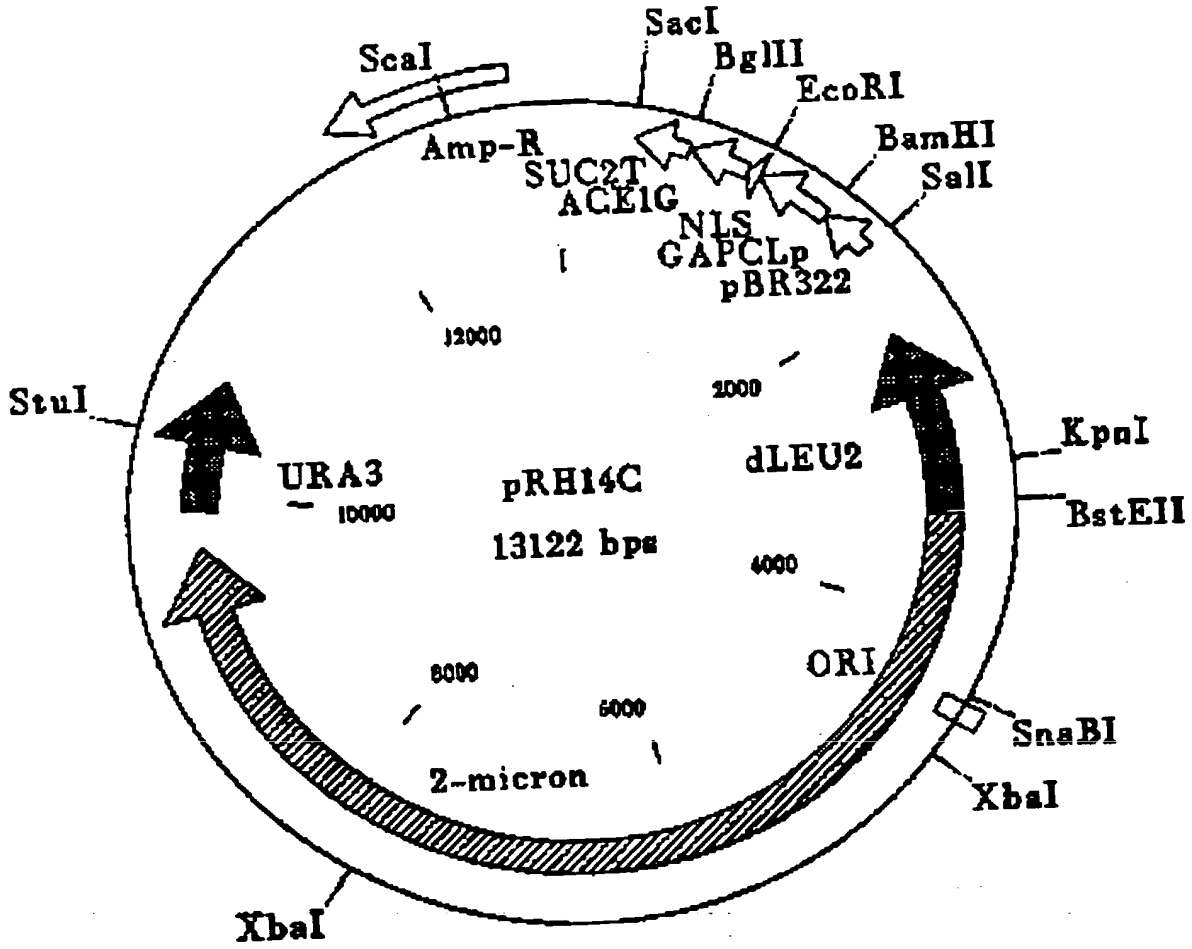
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Fig. 3

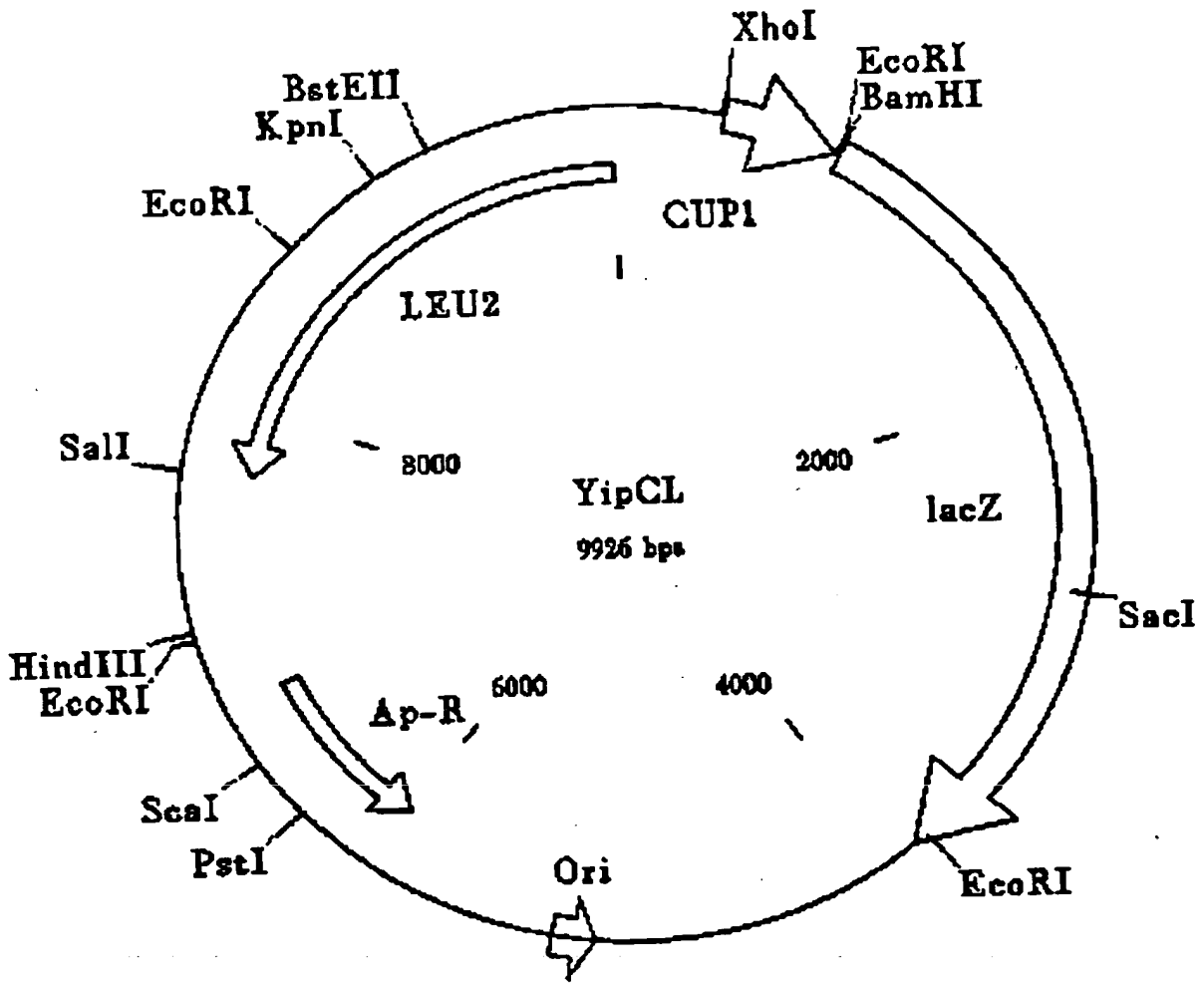
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Fig. 4

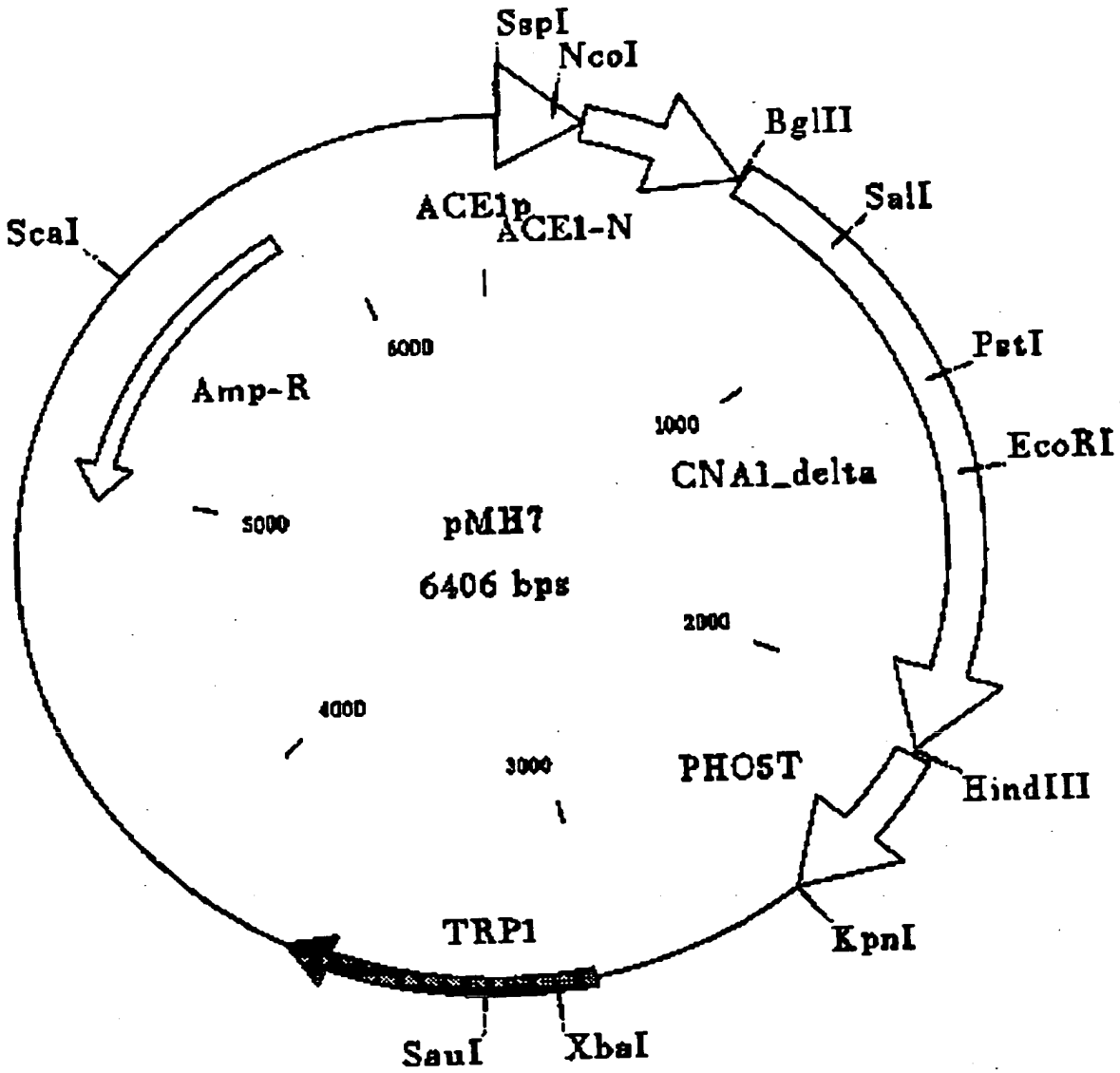
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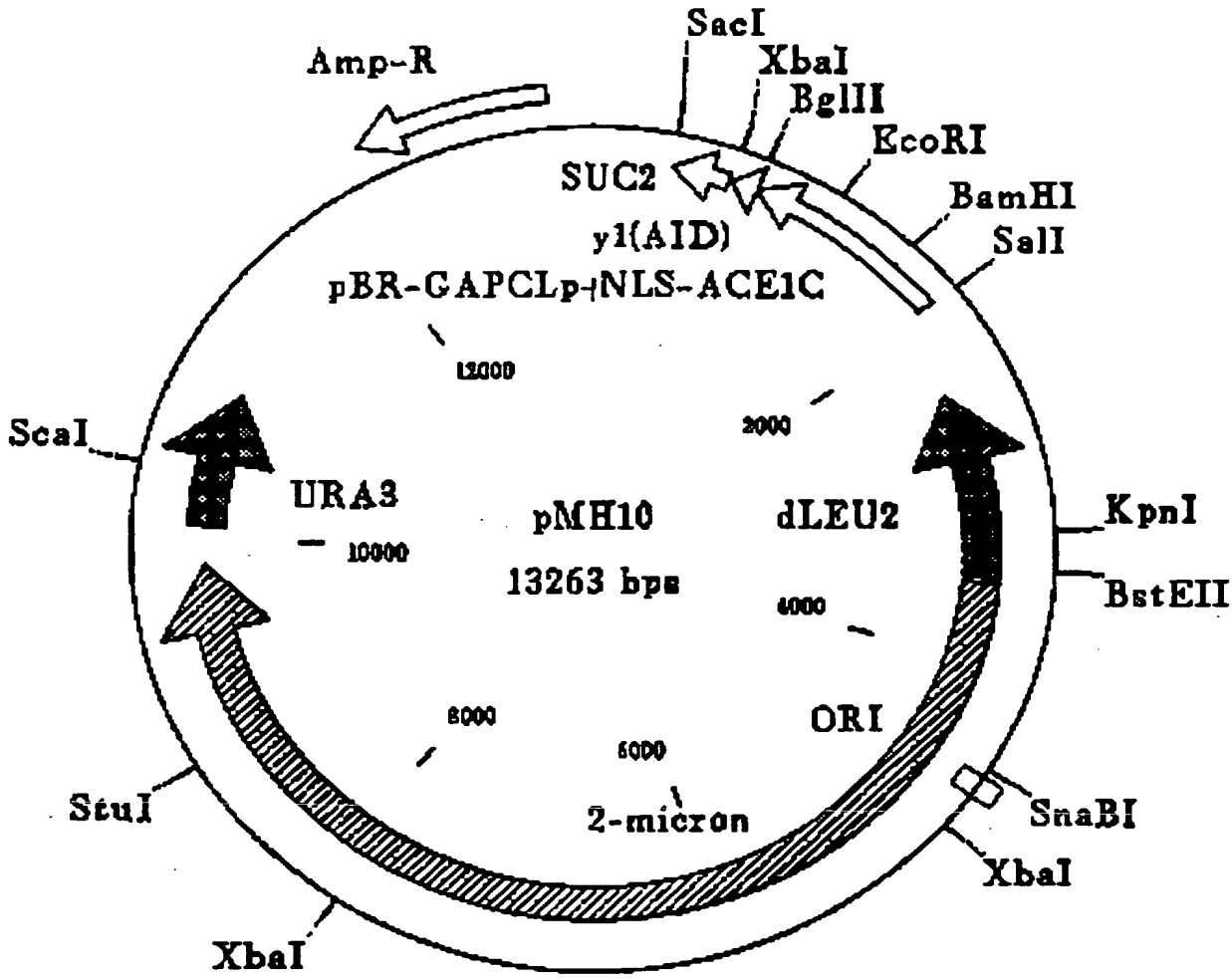
Fig. 5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